STUDIES ON PLASMIDS DETERMINING BACTERIAL HALOACETATE DEHALOGENASES(Dissertation_全文)

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INTRODUCTION

Over the last several decades, a variety of man-made halogenated organic compounds have been released into the environment in large amounts as agricultural and industrial chemicals. The halogen atoms in such organic molecules renders them toxic for microorganisms, insects, pests, weeds, and human beings (1). Since the number of naturally-occurring compounds having carbon-halogen bonds is very limited, microorganisms in nature are limited in their ability to attack these synthetic halogenated compounds (2), which persist and create enormous problems of toxic chemical pollution. Several years ago, the use of many toxic, persistent chlorinated herbicides and pesticides was limited or prohibited by law in Japan and many other countries. Unfortunately, it has recently been pointed out that the toxic chemicals abandoned at that time are now leaking from waste dump sites and contaminating the ground and rivers. These chemicals, which have been called "delayed action chemical bombs," are now becoming a serious problem to human society.

Many chlorinated compounds are slowly degraded by soil microorganisms by co-oxidative metabolism (3, 4). However, there is still no evidence that pure cultures have degraded highly chlorinated compounds such as polychlorinated biphenyl (PCB) (5, 6), while simple chlorinated compounds such as 3-chlorobenzoate and 4-chlorophenoxyacetate are known to be degraded by pure cultures (7-10). Various chlorinated short-chain fatty acids are also degraded by many soil bacteria (11-18). Jensen (11-13) isolated several soil microorganisms capable of degrading chlorinated acetate and propionate, and suggested the presence of an enzyme system

hydrolyzing the carbon-halogen bond. However, little was known of microorganisms that degrade fluorinated compounds.

Fluoroacetate, which has been found in nature only as a toxic component in a plant, Gifblaar, in South Africa (19), is known for its toxic action by the "lethal synthesis" of fluorocitrate, a potent inhibitor of the Krebs cycle enzyme aconitase (20). The compound is extremely toxic to animals, so that it has been used for control of noxious animals such as rats, rabbits, and opossums in Australia and New Zealand. The fluorine-carbon bond in fluoroacetate is chemically much more stable than other halogen-carbon bonds (21) and withstands boiling, concentrated sulfuric acid (22). About twenty years ago, Horiuchi (23), Kelly (24), Goldman (25), and Tonomura et al.(26) isolated Pseudomonas strains that utilize fluoroacetate as a sole source of carbon, with evidence of the occurrence of a enzyme which cleaves the carbon-fluorine bond in accordance with the reaction formula $XCH_2COOH + H_2O \longrightarrow HX + HOCH_2COOH$ (X: halogen).

After that, several dehalogenases with different specificities were found in various microorganisms (27-33), and classified into two groups. One is called haloacetate halidohydrolase (29) [EC 3.8.1.3, the trivial name is haloacetate dehalogenase] which acts specifically on halogen-substituted acetates, and the other is 2-haloacid dehalogenase [EC 3.8.1.2] which has broad specificity to act on 2-halo-substituted short-chain fatty acids such as 2-chloropropionate besides haloacetate (30-33). Although these dehalogenases can all dechlorinate, only a few kinds of haloacetate dehalogenase (23, 25, 26) can defluorinate. Since these dehalogenases mineralize organic halides, microorganisms having dehalogenases may be important in environmental cleaning.

In general, the emergence of microorganisms that degrade a man-made compound may occur by two principal modes (34). The enzyme systems responsible for degradation of naturally-occurring compounds often have broad specificities and may act on synthetic compounds as well as the natural substrates. Alternatively enzymes that act specifically on man-made compounds that are structurally analogous to the natural substrates may evolve from the natural enzymes. Enzyme evolution is initiated by duplication of a gene, then mutation of either gene copy, resulting in the creation of a modified enzyme which acts on novel substrates (35). However, there have been only a few reports which definitely describe enzyme evolution associated with gene evolution. A few instances are the amidase of Pseudomonas aeruginosa (36) having a different specificity from the original enzyme, and the nylon-oligomer degrading enzyme of Flavobacterium sp. (37). Slater et al. (38) have reported the possibility that a 2,2-dichloropropionate-specific dehalogenase has evolved from an original 2-monochloropropionate-specific enzyme in Pseudomonas putida. The diversity of dehalogenases in microorganisms aroused my interest in the evolution of the enzymes acting on man-made halogenated compounds.

It is now well known that bacterial ability to degrade various hydrocarbons and to resist antibiotics are specified by plasmids. Because hydrocarbons such as xylene, toluene, and n-octane are toxic, these degradative plasmids, in analogy with antibiotic resistance plasmids, are believed to be important in the rapid spread of hydrocarbon degradative competence, enabling bacteria to detoxify such compounds (39). In the same way, it was thought that dehalogenase genes which contribute to detoxifying halogenated compounds may occur in plasmids in nature.

Recently, several plasmids involved in biodegradation of chlorinated aromatic compounds have been found in various soil and aquatic bacteria. Pemberton *et al.*(40-42) have demonstrated the occurrence of 2,4-D plasmids (pJP1, pJP2, and pJP3) in *Alcaligenes* spp., which specify bioconversion of 2,4-dichlorophenoxyacetate (2,4-D) to 2,4-dichlorophenol. Plasmids pAC21 and pKF1, which were found in *Klebsiella* and *Acinetobacter* respectively by Chakrabarty *et al.*(43, 44), allow conversion of 4-chlorobiphenyl to 4-chlorobenzoic acid. While these five plasmids specify only part of the degradation without dechlorination, plasmids pAC25 and pB13, which were found in *Pseudomonas* by Chatterjee *et al.*(8, 45) and Reineke *et al.*(46), allow the cells to totally degrade 3-chlorobenzoic acid with the release of chlorine.

Before the finding of pAC25 and pB13, my co-workers and I first found a plasmid specifying dehalogenation. This plasmid, pUO1, has been confirmed to encode two kinds of haloacetate dehalogenase. I have studied the structure and functions of this plasmid for its use in removal of environmental pollutants and also with interest in the evolution of dehalogenase genes.

In this thesis, I describe enzymological studies of the haloacetate dehalogenases determined by the plasmid pUO1 and also genetic studies of the plasmid and the dehalogenase genes. The first chapter deals with the purification and properties of two haloacetate dehalogenases, H-1 from *Pseudomonas* sp. A and H-2 from *Moraxella* sp. B. In the second chapter, the structure and functions of plasmid pUO1 are described, and the cloning of and the properties of the dehalogenase genes of pUO1 are reported in the third chapter. The final chapter describes the frequent occurrence of *in vivo* rearrangement of plasmid pUO1.

CHAPTER I

HALOACETATE DEHALOGENASES OF BACTERIA

Section 1
Isolation of Haloacetate-Assimilating Bacteria

There have been many reports on the microbial degradation of various chlorinated aliphatic acids, such as mono- and dichloroacetate (11-13) and 2-mono- and 2,2-dichloropropionate (14-18). Among them, there are few reports of microorganisms that can degrade fluorinated compounds. Fluoroacetate, one of the simplest halogenated organic compounds, is extremely toxic. The toxicity is due to the inability of organisms to cleave the carbon-fluorine bond, which is much more stable than other halogen-carbon bonds (21).

In the 1960's, some Pseudomonas strains that could utilize fluoroacetate as a sole carbon source were isolated, and the enzymatic release of fluorine from fluoroacetate was demonstrated (23-26). Later, Walker et al. (28) isolated many soil bacteria with fluoroacetate defluorinating activity from New Zealand soils contaminated by fluoroacetate. The number of enzymological studies on dehalogenases that cleave the halogen-carbon bonds in haloacetate or halopropionate are very few, and genetic studies almost non-existent. So, I started with the isolation of fluoroacetate-assimilating bacteria.

MATERIALS AND METHODS

Media and culture conditions. The medium for screening for fluoro- or chloroacetate-assimilable bacteria was

composed of 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% yeast extract, and 0.2% sodium monofluoroacetate (in which case it was called FA medium) or sodium monochloroacetate (CA medium). The pH was adjusted to 7.3 before autoclaving. On occasion, haloacetate was separately sterilized by filtration and then mixed with the autoclaved solution containing other components. Minimal media were made without yeast extract. Peptone medium contained 1% peptone instead of haloacetate. Solid media contained 1.5% agar. Organisms were cultured with shaking at 30°C in a test tube containing 5 ml of the medium or in a Sakaguchi flask containing 100 ml of the medium.

Identification of bacteria. The bacteria isolated were identified according to the 8th edition of Bergey's Manual of Determinative Bacteriology (47).

Determination of minimal inhibitory concentrations (MIC) of drugs. Microorganisms were grown for 3 days with shaking in peptone medium supplemented with antibiotics or mercuric chloride in serial dilution, and the minimal concentration that completely inhibited growth was taken to be the MIC.

DEAE-cellulose column chromatography. Cells grown on fluoroacetate were suspended in 0.02 M phosphate buffer containing 5 mM 2-mercaptoethanol (pH 7.0) and ruptured with an ultrasonic disintegrator (20 kHz). The sonicate was centrifuged at 15,000 x g for 20 min to remove the cell debris, and dialyzed against the buffer overnight. The cell-free extract was put on a DEAE-cellulose column (1.6 x 10 cm) equilibrated with the buffer, and eluted with 100 ml of

linear-gradient phosphate buffer from 0.02 M to 0.3 M.

Assay of haloacetate dehalogenase. The assay system contained 0.2 ml of 0.1 M Tris-HCl buffer (pH 9.0), 0.1 ml of 0.1 M fluoroacetate or chloroacetate, and appropriately diluted enzyme solution in a total volume of 0.5 ml. After a 20-min incubation at 30°C, the reaction was stopped by adding 0.5 ml of 10% trichloroacetic acid, followed by centrifugation if necessary. The colorimetric method of Dagley and Rodgers (48) was used to measure glycolate in a 0.2 ml aliquot. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glycolate per min under these conditions. Protein was determined by the method of Lowry et al. (49) using bovine serum albumin as the standard.

Assay of mercuric reductase and β-lactamase. Activity of mercuric reductase was assayed by measuring $HgCl_2$-dependent NADPH oxidation using the method of Izaki (50). Activity of β-lactamase was measured by the iodometric method of Sawai and Takahashi (51). One unit of enzyme activity was defined as the amount of enzyme oxidizing 1 μmol of NADPH or cleaving 1 μmol of penicillin G per min.

RESULTS AND DISCUSSION

Isolation of fluoroacetate-assimilating bacteria

Several samples of industrial waste water contaminated with fluoroacetate or fluoropropionate and many field-soil and ditch-mud samples from near the University of Osaka Prefecture were used as sources for screening. After enrichment cultivation in the FA or CA medium, microorganisms

were purified on FA or CA plates. Three strains that could utilize fluoroacetate as the sole carbon source were isolated from the sources contaminated with fluorinated compounds; a number of chloroacetate-assimilating bacteria were isolated from the field-soil and ditch-mud. The fluoroacetate-assimilating strains were named A, B, and C, and were further studied.

Taxonomical properties of fluoroacetate-assimilating strains

The morphological and physiological properties of strains A, B, and C are summarized in Table I, II, and III, respectively. According to the Bergey's Manual, I decided that strains A and C were of the genus Pseudomonas, while strain B was either of the genus Moraxella or Acinetobacter. Criteria distinguishing these are that Moraxella is positive

TABLE I. Taxonomical Properties of Strain A

Rods (1.0 by 2.0 μm); motile by polar flagella; no spore formed.
Gram negative; diffusible greenish yellow pigment produced.
Metabolism oxidative; denitrification negative.
Growth factor not required; carbohydrates except glucose not suitable as growth substrates.
Acetate, fluoroacetate, fluoroacetamide, chloroacetate, pyruvate, citrate, succinate, malate, glycolate, glyoxylate, ethanol, glycerol, gluconate and camphor assimilated.
Phenol, salicylate, p-aminobenzoate, naphthalene, kerosine, octane, methanol and formate not assimilated.
Ammonium salts, nitrate salts and amino acids utilized as nitrogen sources; nitrite formed from nitrate; gelatin not liquefied.
Urease present; oxidase positive; catalase positive.
Indol and H_2S not produced; acetyl methyl carbinol test positive.
Strict aerobe.

TABLE II. Taxonomical Properties of Strain B

Rods (1.0-1.5 by 1.5-2.5 μm), in pairs and short chains; no spore formed; flagella not present; capsules present.
Gram negative.
Carbohydrates not utilized.
Acetate, fluoroacetate, fluoroacetamide, chloroacetate, pyruvate, citrate, succinate, malate, glycolate, glyoxylate, butyrate, ethanol, glycerol and camphor assimilated.
Methanol, propionate, octane, kerosine, salicylate, toluene and naphthalene not assimilated.
Ammonium salts, nitrate salts and amino acids utilized as nitrogen sources; nitrite formed from nitrate; gelatin not liquefied.
Urease present; oxidase positive; catalase positive.
Indole and H_2S not produced.
Resistant to penicillin.
Strict aerobe.

TABLE III. Taxonomical Properties of Strain C

Rods (0.6-0.8 by 2.0-3.0 μm); motile by polar flagella; no spore formed.
Gram negative; diffusible yellow pigment produced.
Metabolism respiratory; denitrification positive.
Growth factors not required; carbohydrates not suitable as growth substrates.
Acetate, fluoroacetate, chloroacetate, pyruvate, citrate, succinate, malate, glycolate, glyoxylate, gluconate, ethanol, benzoate and p-hydroxybenzoate assimilated.
Formate, glycerol, fluoroacetamide, methanol, phenol, camphor and salicylate not assimilated.
Ammonium salts, nitrate salts and amino acids utilized as nitrogen sources; gelatine not liquefied.
Urease present; oxidase positive; catalase positive.
Indol and H_2S not produced; acetyl methyl carbinol test negative.
Strict aerobe.

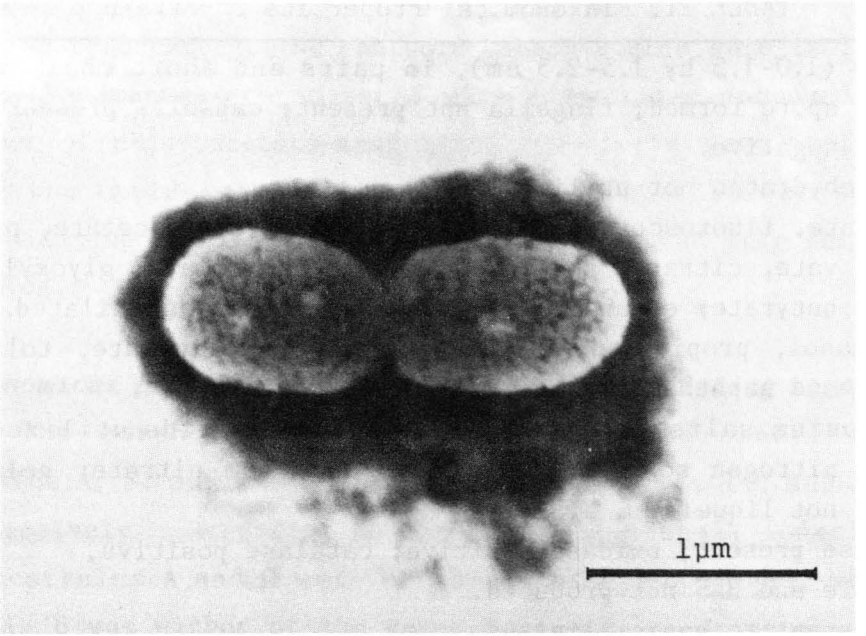


Fig. 1. Electron Micrograph of Moraxella sp. B.

in the oxidase test, but Acinetobacter negative, and that the former is sensitive to penicillin, but the latter resistant. Strain B was positive in the oxidase test and resistant to penicillin; I tentatively classified it as Moraxella, since it resembled Moraxella osloensis. Figure 1 shows an electron micrograph of a pair of strain B cells.

Drug resistance of strains A, B, and C

The MIC of strains A, B, and C against mercuric chloride and several antibiotics are given in Table IV. All three strains were resistant to mercury. Incidentally, I should remark that most chloroacetate-assimilating strains isolated in this laboratory are resistant to mercury. This suggests an interesting relationship between the dehalogenating function and mercury resistance. When these organisms were grown with mercuric chloride, they produced NADP-dependent mercuric reductase in high levels (about 0.09 unit per mg

protein in each cell-free extract). The enzymes were purified and their properties examined. These enzymes resembled each other and also resembled to enzymes from Escherichia coli (50, 52) and Pseudomonas K62 (53).

Strain A was resistant to penicillin and streptomycin, and strain C to nalidixic acid, while strain B was resistant to many antibiotics, including penicillins, streptomycin, kanamycin, gentamycin, rifampicin, and nalidixic acid. Strain B could utilize penicillin G as the sole source of carbon and energy, and cells grown with penicillin G had strong activity of Tem type β-lactamase (0.7 unit per mg protein), but no activity of penicillin acylase.

TABLE IV. Resistant Properties of Strains A, B and C Against Mercury and Antibiotics

Drug	Minimum inhibitory concentration (μg/ml)*		
	Strain A	Strain B	Strain C
$HgCl_2$	50	25	300
Penicillin G	10^3	10^4	10
Carbenicillin	100	>100	-
Oxacillin	-	$>10^3$	-
Streptomycin	10^4	10^4	3
Kanamycin	25	10^4	3
Gentamicin	10	>100	3
Tetracycline	5	5	10
Rifampicin	-	>500	3
Nalidixic acid	-	100	>100

* Culture conditions; in the peptone medium at 30°C with shaking.

Two kinds of haloacetate dehalogenase in fluoroacetate-assimilating bacteria

Strains A, B, and C could assimilate chloroacetate as well as fluoroacetate, and their cell-free extracts had very powerful dehalogenating activity that converted both haloacetates to glycolate, releasing fluorine and chlorine ions. This suggested the presence of haloacetate dehalogenase in these strains.

The dehalogenase activity of cells grown in FA, CA, and peptone media was measured using fluoroacetate and chloroacetate as substrates. The results (Table V) indicate that the dehalogenase of strain A was more active against fluoroacetate than against chloroacetate, and that the strength of the enzyme activity was not much affected by the growth

TABLE V. Effects of Growth Substrates on Dehalogenase Activities of Strains A, B and C.

Strain	Growth substrate	Dehalogenase activity* toward		Ratio of a to b
		Fluoroacetate[a]	Chloroacetate[b]	
A	Fluoroacetate	1.21	0.28	4.3
	Chloroacetate	1.43	0.35	4.1
	Peptone	1.02	0.26	4.0
B	Fluoroacetate	1.50	1.33	1.1
	Chloroacetate	0.51	1.92	0.26
	Peptone	0.05	0.68	0.07
C	Fluoroacetate	0.61	4.92	0.12
	Chloroacetate	0.38	3.28	0.12
	Peptone	0.63	3.63	0.17

* unit per mg protein of cell-free extract.

substrates. Strain C always had higher dechlorinating than defluorinating activity, and the growth substrate had little effect on the activities. However, in the case of strain B, the defluorinating activity was markedly influenced by the growth substrate; thus, the ratio of defluorinating to dechlorinating activity varied with the growth substrate. Such changes of the activity ratio suggested the coexistence of two or more kinds of dehalogenating enzymes in the organism.

To see if such was the case, cell-free extracts of these strains grown on fluoroacetate were analyzed using DEAE-cellulose column chromatography. The chromatograms of strains B and C (Fig. 2) both gave two distinct peaks of

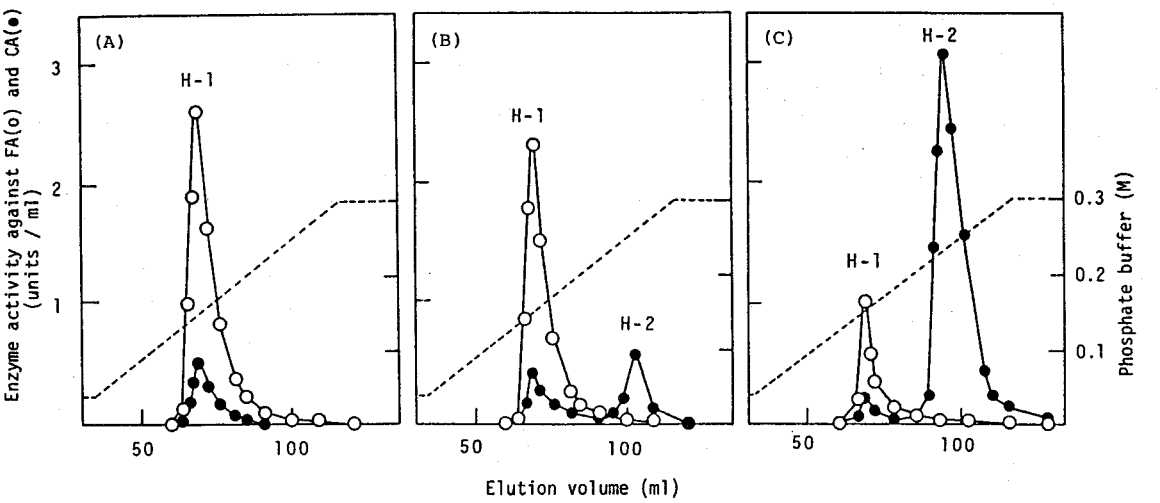


Fig. 2. Chromatograms of Haloacetate Dehalogenases from Pseudomonas sp. A, Moraxella sp. B and Pseudomonas sp. C on DEAE-Cellulose Columns.

(A), Pseudomonas sp. A; (B), Moraxella sp. B; (C), Pseudomonas sp. C. Crude extracts prepared from fluoroacetate-grown cells were applied on the columns (1.6 x 10 cm) equilibrated with 0.02 M phosphate buffer, and eluted with 100 ml of a linear gradient phosphate buffer from 0.02 to 0.3 M (----), collecting 1 ml per tube.

TABLE VI. Substrate Specificity of Dehalogenases Fractionated by DEAE-Cellulose Column Chromatography.

Substrate[a]	Relative activity[b]				
	Strain A	Strain B		Strain C	
		1st-peak	2nd-peak	1st-peak	2nd-peak
Fluoroacetate	5.1	5.4	0	5.1	0
Chloroacetate	1	1	1	1	1
Bromoacetate	0.7	0.7	1.5	0.6	1.4
Iodoacetate	0.00	0.07	1.4	0.06	1.5

a At the concentration of 20 mM.

b Ratio to the activity toward chloroacetate.

dehalogenase activity; the first peaks were active toward fluoroacetate and chloroacetate, while the second ones were active toward chloroacetate only. The extract of strain A gave one peak alone, which was active toward both haloacetates, like the first peaks of strains B and C.

The peak of strain A and the first peaks of strains B and C were at the same retention volume, and the same was true for the second peaks of strains B and C. In addition, the corresponding peaks of each chromatogram showed the same substrate specificities (Table VI). These data mean that the corresponding enzymes of these strains are either very similar or identical. The first-peak enzymes that act preferentially on fluoroacetate were designated H-1 and the second-peak enzymes that do not act on fluoroacetate were designated H-2. The enzymes of strains A and C were all produced constitutively, while the H-1 of strain B was induced by fluoroacetate, and the H-2 seemed to be produced constitutively.

Metabolism of fluoroacetate in strains A and B

The metabolism of fluoroacetate begins with its dehalogenation for conversion to glycolate. Various enzymes known to be involved in glycolate metabolism in microorganisms were searched for in the cell-free extracts of strains A and B grown on fluoroacetate. When fluoroacetate or glycolate was incubated with the cell-free extract, 2,6-dichlorophenolindophenol, and phenazine methosulfate, the substrates were converted to glycolate. Addition of thiamine pyrophosphate and magnesium resulted in the accumulation of glycerate.

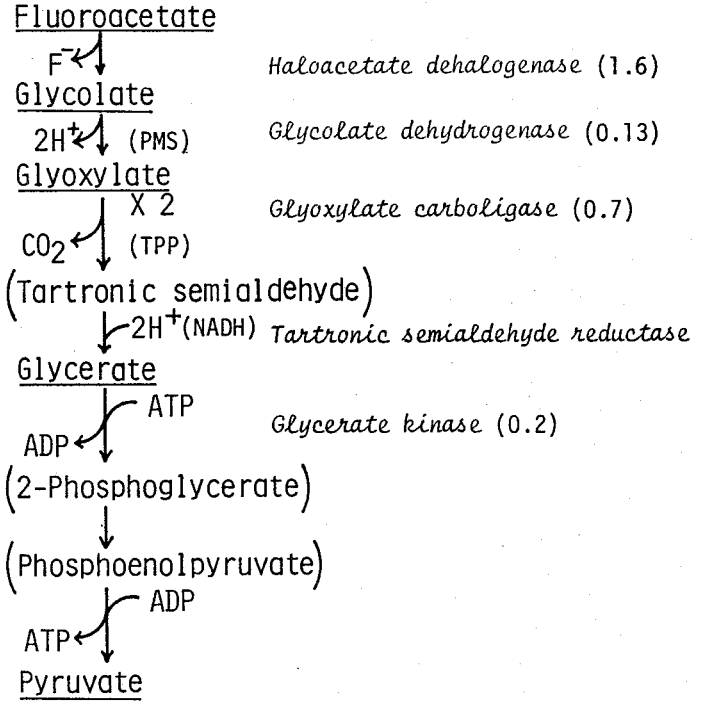


Fig. 3. Tentative Pathway of Fluoroacetate Metabolism in Pseudomonas sp. A and Moraxella sp. B.

Metabolites in parentheses were not identified. Values in parentheses indicate the specific activities (unit/mg protein in cell-free extract) of Pseudomonas enzymes.
PMS, phenazine methosulfate; TPP, thiamine pyrophosphate.

When ATP was added, glycerate was converted to pyruvate. The enzymes involved in these reactions were all inducible by fluoroacetate and were almost absent in cells grown on peptone. From these observations, a tentative pathway of fluoroacetate metabolism was proposed, and shown in Fig. 3. In both strains, dehalogenase activity was far greater than the activities of other enzymes, suggesting that dehalogenase is important in the detoxification of haloacetate.

SUMMARY

Three bacterial strains, Pseudomonas sp. A, Moraxella sp. B, and Pseudomonas sp. C, capable of utilizing fluoroacetate and chloroacetate as the sole carbon source were isolated from industrial waste water contaminated by fluorinated compounds. They were all resistant to mercury and had very powerful dehalogenase activity. Pseudomonas sp. A contained a haloacetate dehalogenase, H-1, that acted on fluoroacetate and chloroacetate, while Moraxella sp. B and Pseudomonas sp. C possessed another dehalogenase, H-2, in addition; H-2 acted on chloro-, bromo-, and iodoacetates but not on fluoroacetate. A tentative pathway of fluoroacetate metabolism in strains A and B was drawn. The enzymes involved in the pathway were all induced by fluoroacetate.

Section 2

Purification and Properties of Haloacetate Dehalogenase H-2 from Moraxella sp. B

Haloacetate dehalogenase, which catalyses the hydrolytic release of halogen from haloacetate to produce glycolate, is classified into two types depending on its reactivity against fluoroacetate. The enzymes reported by Horiuchi (23), Goldman (25), and Tonomura (26) were active toward fluoroacetate, while those of Davies (27), Goldman (31), and Little (32) were inactive. A few of these enzymes have been purified and characterized enzymologically but not physicochemically, in much detail.

In the previous section, I mentioned that Moraxella sp. B has both types of haloacetate dehalogenases, which I called H-1 and H-2; H-1 acts on fluoroacetate but H-2 does not. H-1 was induced by fluoroacetate; H-2 was produced constitutively. In this section, I will describe the purification of H-2 from Moraxella sp. B and then the enzymological and physicochemical properties of the purified enzyme.

MATERIALS AND METHODS

Microorganism and culture. Moraxella sp. strain B, described in the previous section, was cultured aerobically in 7-liter batches for 40 hr at 30°C. The medium was composed of 1% Polypepton, 0.5% sodium monochloroacetate, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4.7H_2O$, and 0.01% yeast extract; the pH was adjusted to 7.5 before autoclaving.

Assay of haloacetate dehalogenase activity. Enzyme

activity was assayed by determining glycolate produced from chloroacetate under the conditions described in Section 1. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol of glycolate per min. The activity of H-2 toward various haloacetates was measured in tne same manner. Activity toward various chlorinated compounds was assayed by determining chloride ions released by the colorimetric method of Okutani (54). In this case, the enzyme reaction was in glycine-NaOH buffer, pH 9.0, instead of Tris-HCl buffer.

Disc electrophoresis. Conventional disc gel electrophoresis was done using the method of Davis (55). A sample was put on a column of 7.5% polyacrylamide gel (pH 9.4) and allowed to run at a current of 5mA per gel for 40 min in Tris-glycine buffer, pH 8.3. Protein bands were stained with Amido Black 10 B.

Determination of molecular weight. The molecular weight was determined by gel filtration on Sephadex G-100 and also by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. Gel filtration was done at 4°C on a Sephadex G-100 column (1.5 x 60 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol. Proteins were eluted with the same buffer at a flow rate of 4 ml per hr.

SDS-polyacrylamide gel electrophoresis was done by the method of Weber and Osborn (56), using a 10% gel in 0.1% SDS-0.1 M phosphate buffer, pH 7.2, at a current of 7 mA per gel for 5 hr. Before electrophoresis, the protein samples were boiled for 5 min in 0.01 M phosphate buffer, pH 7.2, containing 1% SDS, 25% glycerol, and 1% 2-mercaptoethanol. The

gels were stained by immersion in 0.1% Coomassie Brilliant Blue G250 solution and destained in 7% acetic acid.

Analytical ultracentrifugation. The sedimentation velocity of the purified enzyme was measured using a Beckman Model E analytical ultracentrifuge equipped with schlieren optics and operating at 52,000 rpm at 20°C. The enzyme solutions were made at three different concentrations in 0.05 M phosphate buffer, pH 7.0, containing 0.2 M NaCl and 5 mM 2-mercaptoethanol.

Isoelectric focusing. To estimate the pI value of the purified enzyme, gel disc isoelectric focusing was done on 7.5% polyacrylamide gel containing 2% Ampholine (pH 3.5 - 10), as described by Miyazaki (57). As the anode and cathode solutions, 0.02 M H_3PO_4 and 1 M NaOH were used, and electrofocusing continued for 2 hr at the constant voltage of 200 V with cooling. The gels were stained by immersion in Coomassie Brilliant Blue G250-TCA solution.

Amino acid analysis. Using the method of Crestfield et al. (58), purified enzyme was hydrolyzed with 6 N HCl at 110°C for 12, 48, or 70 hr after reductive S-carboxymethylation of cysteine and cystine residues. The hydrolysates were analyzed in a Hitachi KLA-5 Amino Acid Analyzer. Tryptophan was separately determined by the spectrophotometric method of Edelhoch (59).

Materials. DEAE-cellulose was obtained from Serva. Hydroxyapatite was prepared following the method of Tiselius et al. (60). Bio-gel P-150 was obtained from Bio-Rad. Sephadex G-100, blue dextran, and an electrophoresis calibra-

tion protein kit were purchased from Pharmacia Fine Chemicals. Ampholine was from LKB and a pI-marker protein kit A from the Oriental Yeast Co. As calibration proteins for the determination of molecular weight, α-chymotrypsinogen, bovine albumin, and cytochrome c were obtained from the Sigma Chemical Co., and ovalbumin and aldolase from Boehringer Mannheim. All other reagents were of the highest grade available from commercial sources.

RESULTS

Purification of dehalogenase H-1 from Moraxella sp. B

All operations were at 1 - 5°C. Unless otherwise stated, the phosphate buffer used in the purification was always pH 7.0 and contained 5 mM 2-mercaptoethanol.

Step 1. Preparation of cell-free extract. Moraxella cells harvested from 35 liters of a 40-hr culture were washed with 0.15 M NaCl solution and suspended in about 1 liter of cold 0.02 M phosphate buffer. The cells were ruptured with an ultrasonic disintegrator at 20 kHz for 15 - 20 min, and the cell debris was removed by centrifugation at 8000 x g for 20 min. The resulting supernatant (about 2 liters) was dialyzed overnight against 0.02 M phosphate buffer.

Step 2. Protamine treatment. To the cell-free extract, a one-tenth volume of 2% protamine sulfate solution was added, and after 30 min of stirring, the precipitate formed was removed by centrifugation at 8000 x g for 10 min.

Step 3. Fractionation with ammonium sulfate. To the supernatant (2.2 liters) was added solid ammonium sulfate to

0.30 saturation with stirring, and the resulting precipitate was removed by centrifugation at 8000 x g for 15 min. The supernatant was brought to 0.60 saturation with additional ammonium sulfate, and the resulting precipitate was collected by centrifugation and dissolved in 500 ml of 0.05 M phosphate buffer. The crude enzyme solution was dialyzed for 48 hr against 0.05 M phosphate buffer.

Step 4. DEAE-cellulose column chromatography. The dialyzed enzyme was put on a DEAE-cellulose column (5 x 48 cm) equilibrated with 0.05 M phosphate buffer. The column was then washed with the same buffer and the enzyme was eluted with 6 liters of a linear gradient of phosphate buffer from 0.05 M to 0.3 M at a flow rate of 86 ml per hr, 20 ml per tube being collected. The active fractions were combined, dialyzed overnight against 0.05 M phosphate buffer, and rechromatographed on a DEAE-cellulose column (3 x 32 cm) in the same way. The active fractions were combined and dialyzed for 48 hr against 0.01 M phosphate buffer.

Step 5. Hydroxyapatite column chromatography. The dialyzed enzyme solution (770 ml) was put on a hydroxyapatite column (4.2 x 25 cm) equilibrated with 0.01 M phosphate buffer. The column was washed with the same buffer, and developed with 2 liters of phosphate buffer with a linear gradient from 0.01 M to 0.1 M. The active fractions were combined (443 ml), concentrated by ammonium sulfate precipitation, and dialyzed overnight against 0.05 M phosphate buffer.

Step 6. Bio-gel P-150 gel filtration. The dialyzed enzyme solution (7 ml) was put on a Bio-gel P-150 column (22

x 89 cm) equilibrated with 0.05 M phosphate buffer, and eluted at a flow rate of 2.6 ml per hr, 3 ml per tube being collected. The active fractions were combined, concentrated, and dialyzed overnight against 0.05 M phosphate buffer. This preparation had a single protein peak coinciding with enzyme activity on repeated gel filtration.

A summary of the purification is in Table I; the enzyme was purified about 200-fold with a yield of 27% from the cell-free extract.

TABLE I. Purification of Dehalogenase H-2 from Moraxella sp. B

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Purification (-fold)	Yield (%)
Cell-free extract	50900	28400	0.56	1.0	100
Protamine	38600	21400	0.55	1.0	75
$(NH_4)_2SO_4$, 30-60%	23500	22700	0.97	1.7	80
1st DEAE-cellulose	1470	16700	11.3	20.2	59
2nd DEAE-cellulose	924	13800	14.9	26.6	48
Hydroxyapatite	212	10800	50.9	90.9	39
Bio-gel P-150	70	7770	111	198	27

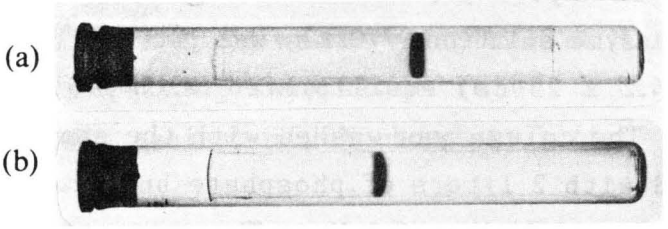


Fig. 1. Disc Gel Electrophoresis of Dehalogenase H-2.

(a) Polyacrylamide gel electrophoresis. Purified enzyme (38 μg) was put on a 7.5% gel column and run at pH 9.4 for 40 min at 5 mA per column. The direction of electrophoresis was from left (cathode) to right (anode).

(b) SDS-Polyacrylamide gel electrophoresis. Purified enzyme (12 μg) treated with SDS at 100°C for 5 min was put on a 10% gel containing 0.1% SDS, and run at 7 mA per column for 5 hr.

Homogeneity of the purified enzyme preparation

Conventional disc gel and SDS-gel electrophoreses of the purified enzyme gave a single protein band (Fig. 1a and b). The sedimentation pattern upon analytical ultracentrifugation (Fig. 2a) also showed the homogeneity of the purified enzyme preparation.

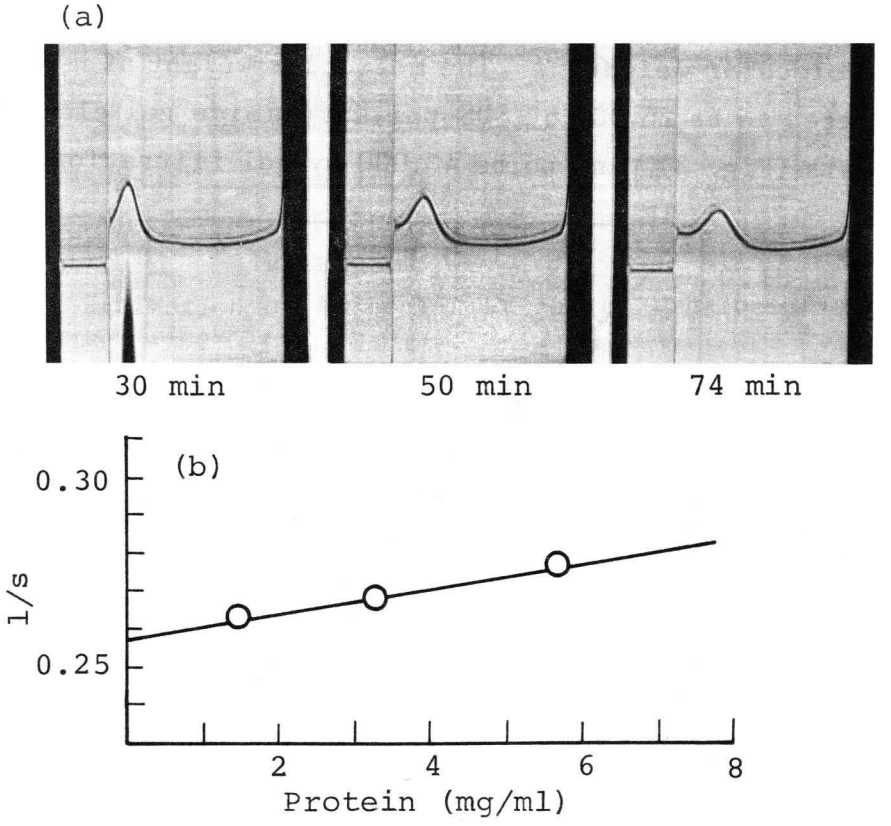


Fig. 2. Ultracentrifugal Analysis of Dehalogenase H-2.

(a) Sedimentation pattern. Purified enzyme (5.7 mg/ml) was centrifuged at 52,000 rpm at 20°C.

(b) Plot of 1/s vs. protein concentrations for determination of the sedimentation coefficient. The enzyme was used at the concentration of 1.5, 3.3, or 5.7 mg of protein per ml of 0.05 M phosphate buffer containing 5mM 2-mercaptoethanol and 0.2 M NaCl.

Physicochemical properties of dehalogenase H-2

Sedimentation coefficient. The purified enzyme was centrifuged under the conditions described in the legend of Fig. 2. By extrapolation of the linear plot of 1/s vs. protein concentrations to the intercept (Fig. 2b), the corrective sedimentation coefficient, $S^{o}_{20,w}$, was calculated to be 4.1 S, using as the value for the partial specific volume of protein, 0.749.

Molecular weight. The molecular weight of H-2 was estimated to be 26,000 by SDS-polyacrylamide gel electrophoresis (Fig. 3), and to be 43,000 by gel filtration on a

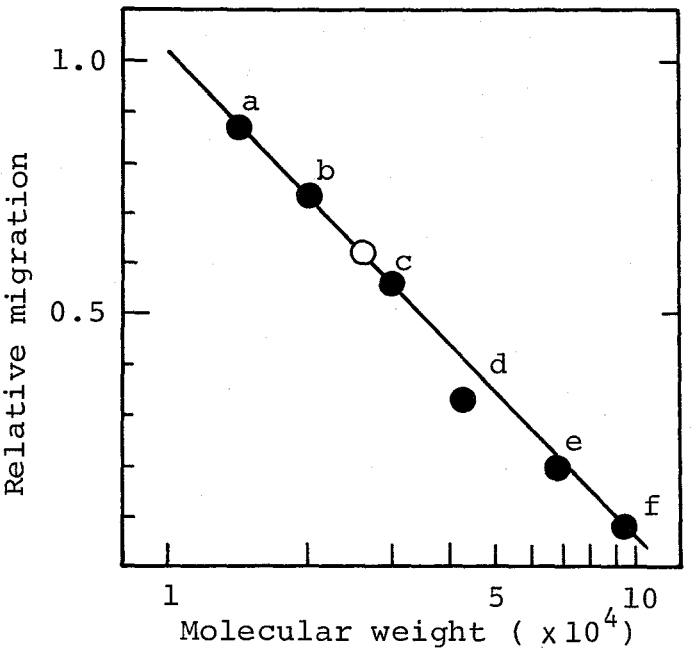


Fig. 3. Estimation of Molecular Weight of Dehalogenase H-2 by SDS-Gel Electrophoresis.

Details of the experiment are described in the text. Standard marker proteins: a, α-lactalbumin (14,400); b, soy bean trypsin inhibitor (20,100); c, carbonic anhydrase (30,000); d, ovalbumin (43,000); e, bovine serum albumin (67,000); f, phosphorylase-b (94,000). The open circle indicates the mobility of dehalogenase H-2.

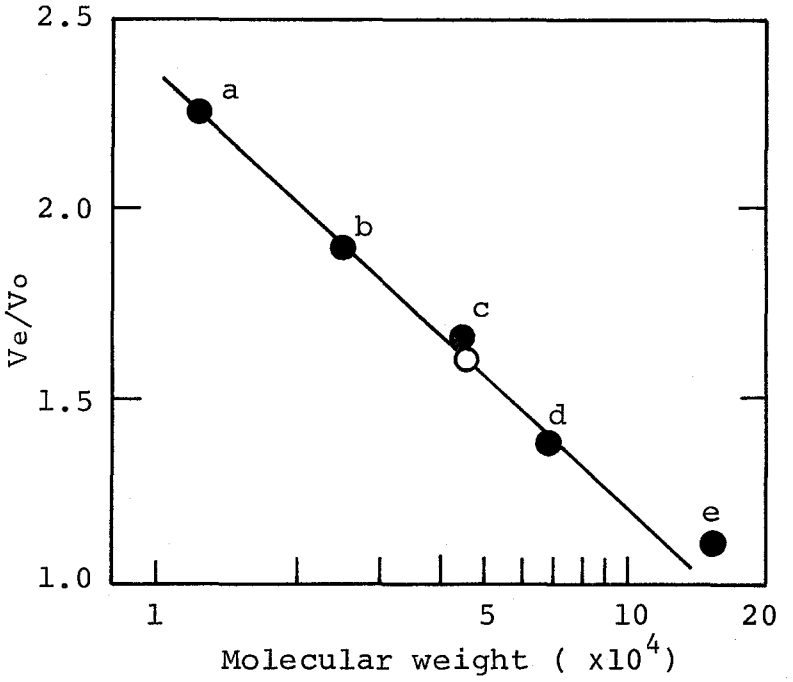


Fig. 4. Estimation of Molecular Weight of Dehalogenase H-2 by Sephadex G-100 Gel Filtration.

Details of the experiment are described in the text. Standard marker proteins: a, cytochrome c (12,500); b, α-chymotrypsinogen A (25,000); c, ovalbumin (45,000); d, bovine albumin (68,000); e, aldolase (158,000). Vo, void volume determined by eluting blue dextran 2000. Ve, elution volume of protein. The open circle indicates the eluting position of dehalogenase H-2.

Sephadex G-100 column (Fig. 4). These values were the averages of three determinations. The difference between the two values exceeds technical error. It seems unlikely that the enzyme protein has subunits structure and exists as a dimer. One possible explanation is that the enzyme is not an ordinary globular protein.

Isoelectric point. Disc electrophoresis on Ampholine-polyacrylamide gel revealed that the H-2 enzyme has an isoelectric point of pH 5.2 (Fig. 5).

Amino acid composition. The purified enzyme was hydrolyzed with HCl and analyzed for amino acid composition. The numbers of amino acid residues shown in Table II were calculated on the basis of a molecular weight of 43,000.

Absorption spectrum. A single absorption peak was observed at 280 nm, but none in the visible region. The extinction coefficient of the purified enzyme at 280 nm ($E^{1\%}_{1cm}$) was 17.76.

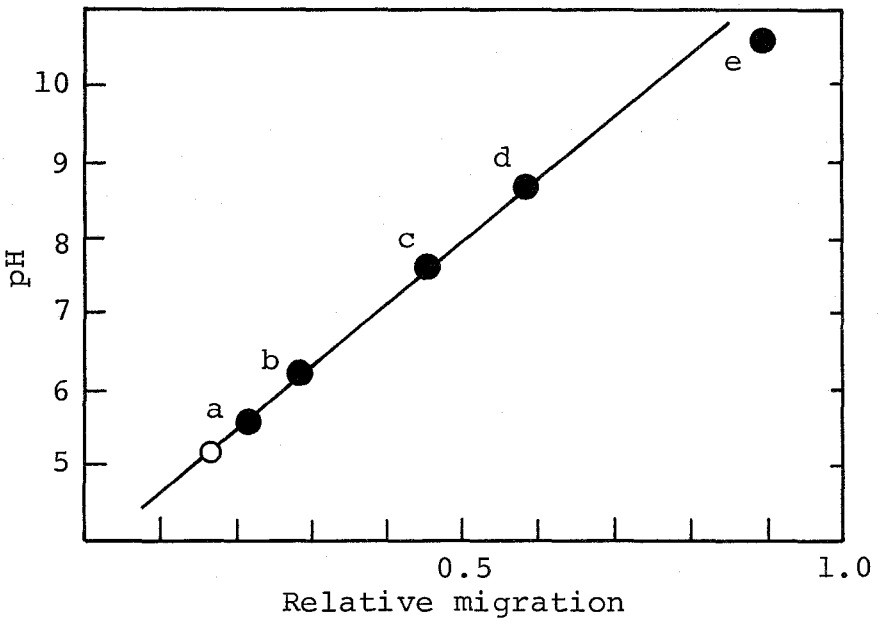


Fig. 5. Estimation of Isoelectric Point of Dehalogenase H-2 by Isoelectric Focusing.

Conditions of electrofocusing were described in the text. pI marker proteins: a, cytochrome c' from Rhodospirillum rubrum (pI 5.6); b, cytochrome c_2 from R. rubrum (pI 6.2); c, myoglobin from horse (pI 7.6); d, myoglobin from whale (pI 8.7); e, cytochrome c from R. rubrum (pI 10.6).

TABLE II. Amino Acid Composition of Dehalogenase H-2

Amino acid	Number of residues per mol of enzyme*1	Amino acid	Number of residues per mol of enzyme*1
Cysteine*2	9.0	Methionine	13.4
Aspartic acid*3	30.6	Leucine	34.6
Threonine*4	14.7	Isoleucine*6	20.5
Serine*4	20.5	Tyrosine	15.7
Glutamic acid*5	40.5	Phenylalanine	16.5
Proline	12.4	Histidine	8.5
Glycine	27.9	Tryptophan*7	8.4
Alanine	34.0	Lysine	20.5
Valine*6	38.1	Arginine	17.4

*1 As the molecular weight of H-2 was adopted 43,000.
*2 Cysteine and cystine were determined as S-carboxymethyl cysteine.
*3 Containing asparagine.
*4 Determined by extraporation to zero time of hydrolysis.
*5 Containing glutamine.
*6 Values obtained after 72-hr hydrolysis.
*7 Determined by the spectrophotometric method (59).

Catalytic properties of dehalogenase H-2

Effects of pH. The optimum pH of the dehalogenase reaction was about 9.5 (Fig. 6a). The enzyme had little activity at acidic pH. Purified enzyme was incubated at various pH for 24 hr at 4°C, and residual activity was assayed. Results (Fig. 6b) indicate that the enzyme is stable between pH 5 and 10.

Effects of temperature. The influence of temperature on the enzyme reaction is shown in Fig. 7a; the highest rate of the reaction was at 50°C. Enzyme dissolved in 0.05 M

phosphate buffer, pH 7, was incubated at various temperatures for 10 min, and the residual activity was measured (Fig. 7b). Enzyme incubated at 60°C retained the initial activity, while the enzyme at 70°C or above lost most of the activity.

Substrate specificity. Dehalogenase H-2 was inactive toward fluoroacetate, even if a large amount of purified enzyme was used in the assay. The enzyme was strongly active against monochloro-, monobromo-, and monoiodoacetate, and the Michaelis constants for these haloacetates were estimated from Lineweaver-Burk plots to be 2.5, 0.5, and 1.1 mM, respectively. These plots also indicated that the haloacetates caused strong substrate inhibition at 10 mM or higher.

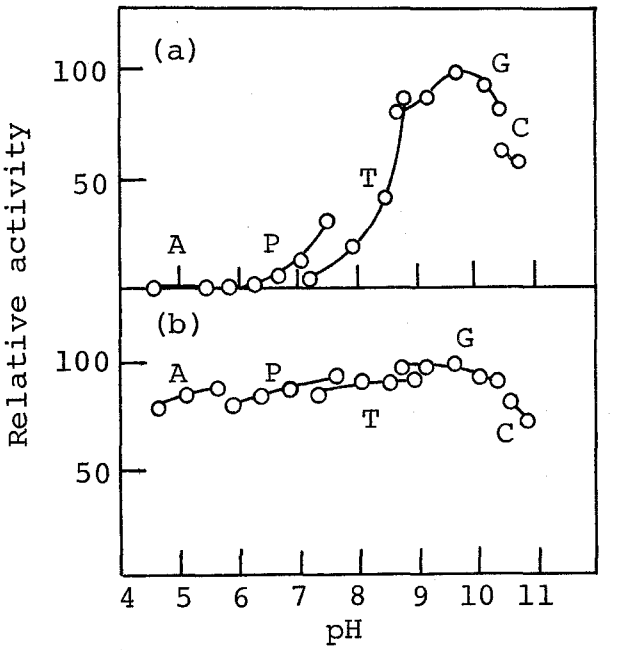


Fig. 6. Effect of pH on Enzyme Activity and Stability.

(a) Optimum pH. The buffers used were as follows. A, sodium acetate-acetic acid; P, KH_2PO_4-Na_2HPO_4; T, Tris-HCl; G, glycine-NaOH; C, sodium carbonate-sodium bicarbonate.

(b) pH Stability. The enzyme was kept at indicated pH for 24 hr at 4°C, and the residual activity was assayed.

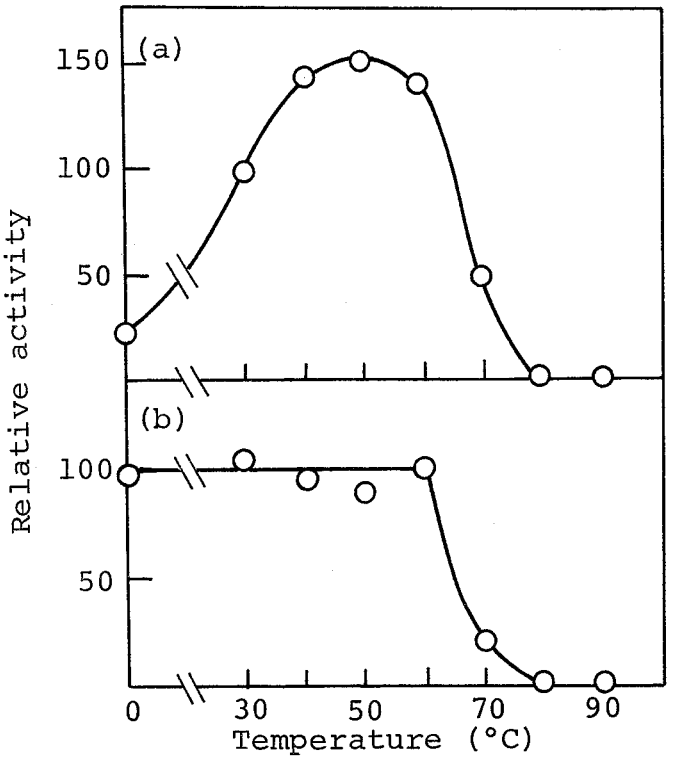


Fig. 7. Effect of Temperature on Enzyme Activity and Stability.

(a) Optimum temperature. Enzyme reaction was allowed to proceed at indicated temperature for 4 min, and glycolate formed was assayed.

(b) Thermostability. The enzyme was incubated for 10 min at indicated temperature, and then the residual activity was assayed.

Activity toward various chlorinated compounds was assayed by measuring the rates of chloride ion release. Table III shows that 2,2-dichloroacetate and 2-chloropropionate were only slightly dehalogenated, but 3-chloropropionate and 2-, 3- and 4-chlorobutyrates were dehalogenated not at all. This means that the H-2 enzyme is a haloacetate dehalogenase rather than a 2-haloacid dehalogenase.

TABLE III. Substrate Specificity of Dehalogenase H-2

Enzyme activities against monohaloacetate were determined by measuring glycolate formed, and those against chlorinated compounds were by measuring chloride ions released. The relative activity was expressed as percentage to the activity for monochloroacetate. Substrate concentration was 2.5 mM.

Substrate	Relative activity (%)
Monofluoroacetate	0.0
Monochloroacetate	100
Monobromoacetate	160
Monoiodoacetate	150
Dichloroacetate	3.3
Trichloroacetate	0.0
2-Chloropropionate	9.5
3-Chloropropionate	0.0
2,2-Dichloropropionate	0.1
2-Chlorobutyrate	0.0
3-Chlorobutyrate	0.0
4-Chlorobutyrate	0.0
Chloroacetamide	0.0
Chloroacetate ethylester	53.3

Inhibitor. The inhibition by several reagents of the H-2 enzyme is shown in Table IV. Thiol reagents such as p-chloromercuribenzoate (pCMB), p-chloromercuriphenyl sulfonic acid (pCMPSA), $HgCl_2$, and $AgCl_2$ inhibited enzyme activity strongly even at low concentrations such as 10^{-6}M. Chelating agents did not cause any inhibition. Dehalogenation of monochloroacetate (2.5 mM) was inhibited by about 20% when 20 mM monofluoroacetate was present, although it was not affected by 10 mM acetate or glycolate.

TABLE IV. Effect of Inhibitors on Dehalogenase H-2

Purified enzyme solution, from which mercaptoethanol was removed by dialysis, was preincubated for 5 min in 25 mM Tris-HCl buffer (pH9) containing inhibitor indicated, and then monochloroacetate was added to it, followed by measurement of enzyme activity.

Inhibitor	mM	Inhibition (%)
pCMB	10^{-3}	97
pCMPSA	10^{-3}	92
N-Ethylmaleimide	1	79
$HgCl_2$	10^{-3}	89
$AgCl_2$	10^{-3}	45
EDTA	10^{-2}	15
o-Phenanthroline	1	0
α,α'-Dipyridyl	1	0

pCMB; p-chloromercuribenzoate.
pCMPSA; p-chloromercuriphenyl sulfonic acid.

DISCUSSION

Bacterial dehalogenases acting on carbon halide in aliphatic acids have been classified into two groups, 2-haloacid dehalogenase [EC 3.8.1.2] and haloacetate dehalogenase [EC 3.8.1.3] (61). In the first group are an Arthrobacter enzyme reported by Kearney (62) and Pseudomonas enzymes reported by Goldman (31), Little (32), and Slater (17). In the second are Pseudomonas enzymes reported by Horiuchi (23), Davies (27), Goldman (25), and Tonomura (26). The H-2 enzyme of the Moraxella sp. B described here can be classified as a haloacetate dehalogenase, since the enzyme is much more active toward all monohaloacetates (except monofluoroacetate) than toward 2-halo substituted propionate or butyrate.

Little et al. (32) have written that there are two kinds of dehalogenases that can be distinguished by sensitivity to thiol reagents, suggesting that there may be two different mechanisms of dehalogenation. The haloacetate dehalogenases of Davies (27) and Goldman (25) are susceptible to thiol reagents, while the 2-haloacid dehalogenases of Little (32) and of Goldman (31) are virtually unaffected. Moraxella enzyme H-2 was extremely sensitive to thiol reagents. It seems that the sensitive enzymes correspond to haloacetate dehalogenase and the insensitive enzymes to 2-haloacid dehalogenase.

Dehalogenases may be also distinguished by reactivity with fluoroacetate, since the fluorine-carbon bond is much more stable than other halogen-carbon bonds. The enzymes of Horiuchi (23), Goldman (25), and Tonomura (26) were active against fluoroacetate, but the enzyme of Davies (27), Goldman (31), and Little (32) were inactive. Moraxella H-2 did not act on fluoroacetate. No 2-haloacid dehalogenases have been found with defluorinating activity.

Moraxella H-2 enzyme resembles the enzyme of Davies (27) in its catalytic properties. Moraxella H-2 is different from the enzyme of Pseudomonas dehalogenans (32), which has been well-characterized enzymologically and physicochemically. The molecular weight of the Pseudomonas enzyme was 15,000, which was estimated by Sephadex G-100 gel filtration, while H-2 has a molecular weight of 43,000. The amino acid compositions of the two enzymes were different also. Although the effects of pH on the enzyme reaction and enzyme stability were almost the same, sensitivity to thiol reagents was different. Moraxella H-2 was much more sensitive. The Pseudomonas enzyme was somewhat active against DL-2-chloropropionate, but Moraxella enzyme was active only slightly.

There are some differences in enzymological and physicochemical properties between haloacetate dehalogenases and 2-haloacid dehalogenases, and also such differences among dehalogenases classified in the same group. It is not certain whether such diversity can be accounted for by variance from a certain original enzyme gene; the evolutionary relationships of these dehalogenases is unknown.

SUMMARY

Here, haloacetate dehalogenase H-2 was purified from fluoroacetate-assimilable Moraxella sp. B. The purification procedures included protamine treatment, ammonium sulfate fractionation, and column chromatography with DEAE-cellulose, hydroxyapatite, and Bio-gel P-150, resulting in a 200-fold purification. The purified enzyme was homogeneous by the criteria of ultracentrifugation and disc electrophoresis.

The molecular weight estimated on Sephadex G-100 gel filtration was 43,000; it was 26,000, estimated using SDS-polyacrylamide gel electrophoresis. The sedimentation coefficient $s^{o}_{20,w}$ was 4.1 S, and the isoelectric point was pH 5.2. The amino acid composition was also calculated.

This enzyme catalyzed the dehalogenation of monochloro-, monobromo-, and monoiodoacetate, but not monofluoroacetate. 2,2-Dichloroacetate and 2-chloropropionate were slightly dehalogenated, but trichloroacetate and 3-chloropropionate were not. The enzyme was very sensitive to inhibition with thiol reagents.

Section 3
Purification and Properties of Haloacetate Dehalogenase H-1 from Pseudomonas sp. A

In the previous section, it was shown that Moraxella sp. B has two haloacetate dehalogenases, H-1 and H-2; H-2 was purified and characterized. Unlike the constitutive H-2 enzyme, the H-1 enzyme was inducibly produced when fluoroacetate was used as the growth substrate. To obtain enough of the enzyme for purification, a large amount of fluoroacetate was needed, which is very toxic to humans. The haloacetate dehalogenase of Pseudomonas sp. A that resembles H-1 was produced constitutively. In this section, the purification and crystallization of the dehalogenase H-1 from Pseudomonas sp. A are described, and its properties are compared with those of the Moraxella H-1 and H-2 enzymes.

MATERIALS AND METHODS

Microorganism and culture. Strain A of Pseudomonas sp. described in Section 1, was used. The organism was aerobically grown in 6 liters of medium containing 1% peptone, 0.1% KH_2PO_4, 0.4% $Na_2HPO_4.12H_2O$, 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4.7H_2O$, and 0.1% yeast extract, pH 7.0.

Assay of haloacetate dehalogenase activity. H-1 activity was assayed using monofluoroacetate as the substrate under the conditions described in the previous section. One unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of glycolate per min. Enzyme activity against other substrates was determined as described in Section 2.

Analytical methods. Disc gel electrophoresis, analytical ultracentrifugation, isoelectric focusing, determinations of molecular weight by Sephadex G-100 gel permeation and SDS gel electrophoresis, and amino acid analysis were done as described in Section 2.

RESULTS

Purification of dehalogenase H-1 from Pseudomonas sp. A

The procedures used for the purification of the Pseudomonas H-1 enzyme were almost the same as those for the Moraxella H-2 enzyme. The buffer used was phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol.

The cells (about 57 g wet weight) were harvested from 6 liters of culture, washed with 0.05 M phosphate buffer, and disrupted using an ultrasonic disintegrator. The cell-free extract obtained was heated at 52.5°C for 5 min, and the precipitate of denatured protein was removed by centrifugation. To the supernatant was added protamine sulfate equivalent to 20% of the total protein present. After removal of the nucleic acids precipitate, the enzyme solution was fractionated with ammonium sulfate and the fraction from 30 to 70% saturation was collected.

The enzyme fraction was dialyzed against 0.05 M phosphate buffer and put on a DEAE-cellulose column (5.1 x 43.5 cm) equilibrated with 0.05 M phosphate buffer. The enzyme was eluted with 2 liters of a linear gradient phosphate buffer from 0.05 to 0.3 M. The active fractions were pooled, dialyzed against 0.01 M phosphate buffer, and put on a hydroxyapatite column (2.0 x 25.5 cm) equilibrated with 0.01 M phosphate buffer, followed by elution with 1 liter of a linear gradient phosphate buffer from 0.01 M to 0.1 M. The

enzyme eluted was precipitated by ammonium sulfate, dissolved in a small volume of 0.05 M phosphate buffer, and put on a Bio-gel P-150 column (2.5 x 60 cm). The active fraction was concentrated by ammonium sulfate precipitation and allowed to crystallize. A summary of the purification is presented in Table I.

TABLE I. Purification of Dehalogenase H-1 from Pseudomonas sp. A

Step	Protein (mg)	Activity (units)	Specific activity (units/mg)	Yield (%)
Cell-free extract	6990	7120	1.02	100
Heat treatment	2660	5330	2.00	75
Protamine	2240	5440	2.43	76
$(NH_4)_2SO_4$, 30-70%	1450	5800	4.00	81
DEAE-Cellulose	483	3960	8.20	56
Hydroxyapatite	131	2960	22.7	42
Bio-gel P-150	66.4	2030	30.6	29
Crystallization	-	-	38.4	-

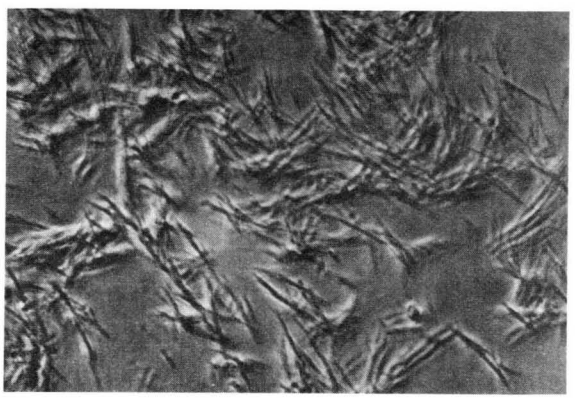


Fig. 1. Crystals of Haloacetate Dehalogenase H-1 of Pseudomonas sp. A.

Crystallization of dehalogenase H-1

To a concentrated enzyme solution (about 10 mg protein per ml), solid ammonium sulfate was gradually added until a faint turbidity appeared. Left at 4°C, needle-shaped crystals formed (Fig. 1). The crystals were collected, dissolved in a small volume of buffer, and recrystallized in the same way. The recrystallized enzyme gave a single band on disc gel electrophoresis (Fig. 2), and also sedimented as a single, symmetric peak on analytical ultracentrifugation (Fig. 3), showing that the purified enzyme was homogeneous.

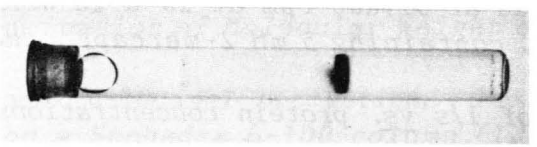


Fig. 2. Polyacrylamide Gel Disc Electrophoresis of Crystalline H-1 enzyme.

Purified enzyme (20 ug) was electrophoresed on a 7.5% gel at pH 9.4 for 40 min at 5mA per column. The direction of electrophoresis was from left (cathode) to right (anode).

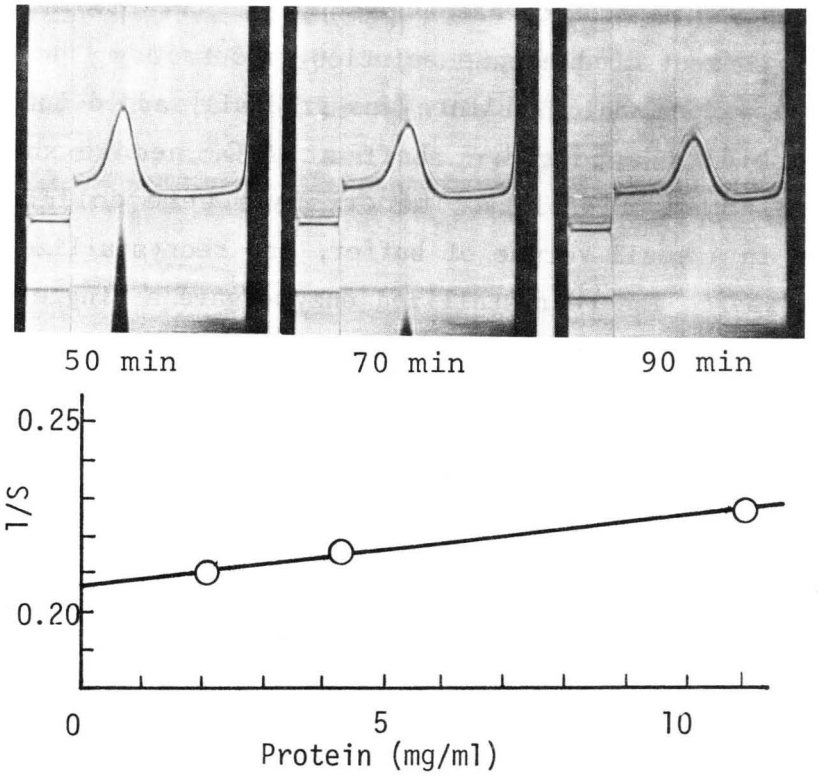


Fig. 3. Ultracentrifugal Analysis of Dehalogenase H-1.

(a) Sedimentation patterns. Purified enzyme (11 mg/ml) was centrifuged at 52,000 rpm at 20°C in 0.05 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol and 0.2 M NaCl.

(b) Plot of 1/s vs. protein concentrations for determination of the sedimentation coefficient. Protein concentrations were 2.1, 4.3, and 11 mg per ml.

Physicochemical properties of dehalogenase H-1

The molecular weight of the enzyme was determined to be 42,000 by Sephadex G-100 gel permeation (Fig. 4a) and 33,000 by SDS-polyacrylamide gel electrophoresis (Fig. 4b). Values by these two methods were different in the case of the Moraxella H-2 enzyme also. The sedimentation coefficient $s^{o}_{20,w}$ was calculated to be 5.2 S from the data in Fig. 3.

The isoelectric point was estimated to be pH 5.4 by isoelectric focusing in Ampholine-polyacrylamide (Fig. 5). The enzyme has a UV absorption spectrum with a maximum at 280 nm and a slight shoulder around 290 nm, but no characteristic absorption in the visible region. The extinction coefficient at 280 nm, $E^{1\%}_{1cm}$, was 15.67. The amino acid composition of the H-1 enzyme is shown in Table II.

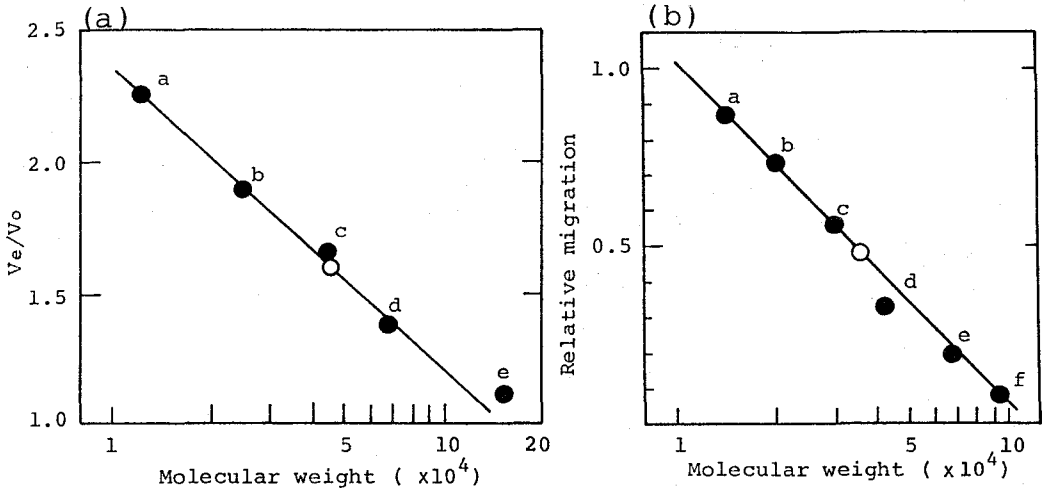


Fig. 4. Determination of Molecular Weight of Dehalogenase H-1.

(a) Sephadex G-100 gel permeation. The permeation was carried out on a Sephadex G-100 column (1.5 x 69 cm) at a flow rate of 4 ml per hr. Standard marker proteins: a, cytochrome c (12,500); b, α-chymotrypsinogen A (25,000); c, ovalbumin (45,000); d, bovine albumin (68,000); e, aldolase (158,000). Vo, void volume determined by eluting blue dextran 2000. Ve, elution volume of protein. The open circle indicateds the eluting position of the H-1 enzyme.

(b) SDS-gel electrophoresis. The SDS-treated enzyme and marker proteins were electrophoresed on a 10% polyacrylamide gel in 0.1% SDS - 0.1 M phosphate buffer, pH 7.2, at 7 mA per gel for 5 hr. Standard marker proteins: a, α-lactalbumin (14,400); b, soy bean trypsin inhibitor (20,100); c, carbonic anhydrase (30,000); d, ovalbumin (43,000); e, bovine serum albumin (67,000); f, phosphorylase-b (94,000). The open circle indicates the mobility of the H-1 enzyme.

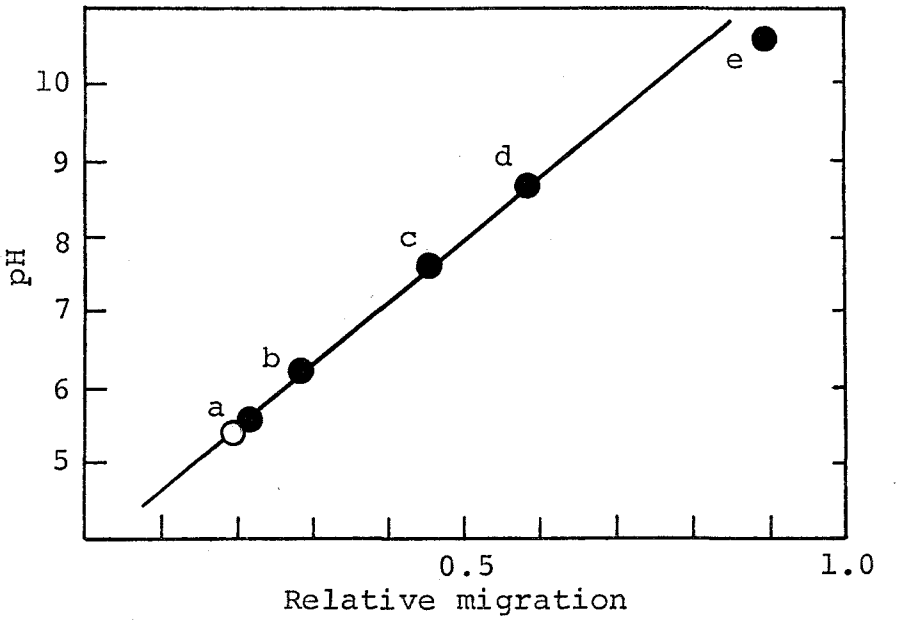


Fig. 5. Determination of Isoelectric Point of Dehalogenase H-1 by Isoelectric Focusing.

Electrofocusing was performed on a 7.5% polyacrylamide gel containing 2% Ampholine (pH 3.5-10) at 200 V for 2 hr with cooling. Marker proteins: a, cytochrome c' of Rhodospirillum rubrum (pI 5.6); b, cytochrome c_2 of R. rubrum (pI 6.2); c, myoglobin of horse (pI 7.6); d, myoglobin of whale (pI 8.7); e, cytochrome c of R. rubrum (pI 10.6).

Catalytic properties of dehalogenase H-1

Effects of pH and temperature. The enzyme had maximum activity at pH 9.0, and was stable in the pH range between 6.0 to 10.0. The highest reaction rate was observed at 50°C. However, at this temperature, 10 min of incubation led to a loss of 50% the enzyme activity.

Substrate specificity. The enzyme had the strongest affinity for monofluoroacetate (Km, 2.0 mM). The Km values for monochloro- and monobromoacetate were 4.8 and 6.5 mM,

TABLE II. Amino Acid Compositions of Dehalogenases H-1 and H-2

The purified enzymes were hydrolyzed with 6 N HCl at 110°C for 12, 48 and 70 hr. As the molecular weights were adopted 42,000 for H-1 and 43,000 for H-2.

Amino acid	Number of residues per mol of enzyme	
	H-1	H-2
Cystein (half cystine)*1	6.0	9.0
Aspartic acid (asparagine)	39.5	30.6
Threonine*2	18.7	14.7
Serine*2	19.2	20.5
Glutamic acid (glutamine)	32.9	40.5
Proline	24.5	12.4
Glycine	30.0	27.9
Alanine	30.5	34.0
Valine*3	28.9	38.1
Methionine	7.9	13.4
Leucine	27.9	34.6
Isoleucine*3	9.8	20.5
Tyrosine	14.9	15.7
Phenylalanine	23.9	16.5
Histidine	15.9	8.5
Tryptophan*4	10.3	8.4
Lysine	12.2	20.5
Arginine	21.7	17.4

*1 Determined as S-carboxymethyl cysteine.
*2 Determined by extraporation to zero time of hydrolysis.
*3 After 72 hr hydrolysis.
*4 Determined by the spectrophotometric method.

respectively. Monoiodoacetate, which was a good substrate for the Moraxella H-2 enzyme, was dehalogenated only slowly. Relative activities against various substrates are shown in Table III. H-1 was much more active toward monohaloacetates than toward 2-halopropionate or -butyrate.

Inhibitors. Thiol reagents such as p-chloromercuribenzoate and $HgCl_2$ markedly inhibited the enzyme (Table IV). The presence of 1 mM glycolate in the reaction mixture reduced by about 70% the dehalogenation velocity against 2.5 mM monochloroacetate.

TABLE III. Substrate Specificity of Dehalogenase H-1

Enzyme activities against monohaloacetate were determined by measuring glycolate formed, and against chlorinated compounds by measuring chloride ions released. The relative activity was expressed as percentage to the activity for monochloroacetate. Substrate concentration was 2.5 mM.

Substrate	Relative activity (%)
Monofluoroacetate	510
Monochloroacetate	100
Monobromoacetate	70
Monoiodoacetate	0.2
Dichloroacetate	1.4
Trichloroacetate	0.0
2-Chloropropionate	2.6
3-Chloropropionate	< 0.1
2,2-Dichloropropionate	0.0
2-Chlorobutyrate	< 0.1
3-Chlorobutyrate	0.0
4-Chlorobutyrate	0.0
Chloroacetamide	< 0.1

TABLE IV. Effect of Inhibitors on Dehalogenase H-1

Purified enzyme solution, from which mercaptoethanol was removed by dialysis, was preincubated for 5 min in 25 mM Tris-HCl buffer(pH 9) containing inhibitor indicated, and then the reaction was started by an addition of monochloroacetate.

Inhibitor	mM	Inhibition (%)
pCMB	10^{-2}	96
pCMPSA	10^{-2}	92
N-Ethylmaleimide	1	68
$HgCl_2$	10^{-2}	99
$AgCl_2$	10^{-3}	61
EDTA	10^{-2}	0
o-Phenanthroline	1	0
α,α'-Dipyridyl	1	0

pCMB; p-chloromercuribenzoate.
pCMPSA; p-chloromercuriphenyl sulfonic acid.

DISCUSSION

This is the first success in crystallization of dehalogenase. The enzyme of Pseudomonas sp. A is compared here with the partially purified H-1 enzyme of Moraxella sp. B. The two enzymes have an identical molecular weight, 42,000, by Sephadex G-100 gel permeation. The effects of pH and temperature on enzyme activity and stability are also identical. Substrate specificity is almost identical. These similarities suggest that the enzyme of Pseudomonas may be identical with H-1 from Moraxella. In a later section, the homology of the genes of the two enzymes will be mentioned. The H-1 enzymes described here seem to resemble the haloacetate dehalogenase reported by Goldman (25) in their

catalytic properties.

Next, the Pseudomonas H-1 enzyme is compared with the Moraxella H-2 enzyme characterized in Section 2. Substrate specificity of this H-1 and H-2 are different. H-1 acts preferentially on monofluoroacetate, while the H-2 does not, having the most affinity for monobromoacetate. The two enzymes have little activity against chlorinated propionate and butyrate, and are sensitive to thiol reagents. These properties suggest that both enzymes are haloacetate dehalogenases. Some properties of H-1 and H-2 enzymes are summarized in Table V. Catalytic properties are similar except for substrate specificity; properties of the proteins, such as the sedimentation coefficient and the amino acid composition (Table II), are somewhat different. H-1 contains more proline and histidine and less isoleucine and lysine than H-2.

TABLE V. Comparison of Properties of Dehalogenases H-1 and H-2.

Property	H-1	H-2
Molecular weight		
Gel permeation	42,000	43,000
SDS-electrophoresis	33,000	26,000
$s^{o}_{20,w}$	5.2 S	4.1 S
$E^{1\%}_{280nm}$	15.67	17.76
pI	pH 5.4	pH 5.2
Optimum pH	pH 9.0	pH 9.5
Optimum temperature	50°C	50°C
Stability	pH6 - 10	pH5 - 10
	< 50°C	< 45°C
Inhibition with pCMB	10^{-5} M	10^{-6} M

SUMMARY

Haloacetate dehalogenase H-1 was purified from Pseudomonas sp. A and crystallized. The purification procedures involved ultrasonic rupture, heat treatment, protamine treatment, ammonium sulfate fractionation, column chromatography with DEAE-cellulose, hydroxyapatite, and Bio-gel P-150, and crystallization with ammonium sulfate, resulting in a 38-fold purification. The purified enzyme was homogeneous by the criteria of ultracentrifugation and disc gel electrophoresis.

Physicochemical and catalytic properties of the H-1 enzyme were studied. The molecular weight was 42,000, the sedimentation coefficient $s^{o}_{20,w}$ was 5.2 S, and the isoelectric point was pH 5.4. The amino acid composition was also calculated. The optimum pH and temperature were pH 9.0 and 50°C. The enzyme acted preferentially on monofluoroacetate (Km, 2 mM) but not on 2-halopropionate. Thiol reagents inhibited the enzyme activity markedly. Some properties of the H-1 enzyme were different from those of the H-2 enzyme of Moraxella sp. B.

CHAPTER II

PLASMID PUO1 THAT DETERMINES HALOACETATE DEHALOGENASE IN MORAXELLA SP. B

Section 1
Plasmid-Dependency of the Dehalogenating Capability of Moraxella sp. B

Bacterial ability to degrade some hydrocarbons and to resist antibiotics are specified by plasmids (63). There is also some evidence that plasmids are involved in bacterial adaptation to the surroundings.

Bacterial dehalogenating ability may depend on certain plasmids. In this section, I describe evidence that two haloacetate dehalogenases of Moraxella sp. B, H-1 and H-2, are specified by a plasmid.

MATERIALS AND METHODS

Organisms and culture. Moraxella sp. B and the derivative strains used are listed in Table I. The culture medium contained 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.1%$(NH_4)_2SO_4$, 0.01% $MgSO_4.7H_2O$, 0.01% yeast extract, and 0.2% carbon source, such as sodium acetate, sodium monofluoroacetate, monochloroacetate, casamino acids, or peptone. The minimal media were made without yeast extract. The media were solidified with 1.5% Noble agar (DIFCO). For auxanography the minimal acetate media supplemented with 100 µg of each amino acid per milliliter were used. The organisms were aerobically cultured at 30°C.

TABLE I. Wild and Derivative Strains of Moraxella sp. B

Strain No.	Phenotype	Parent	Treatment
B	$H\text{-}1^+$ $H\text{-}2^+$ Hg^r		
B86	$H\text{-}1^+$ $H\text{-}2^-$ Hg^r	B	Mit C
B123	$H\text{-}1^-$ $H\text{-}2^-$ Hg^s	B	Mit C
B186	$H\text{-}1^+$ $H\text{-}2^+$ Hg^r Ilv^-	B	NTG
B44	$H\text{-}1^+$ $H\text{-}2^-$ Hg^r Ilv^-	B186	
P2	$H\text{-}1^+$ $H\text{-}2^+$ Hg^r	B123	Mating

Abbreviations: Ilv^-, requirement of isoleucine, leucine and valine; NTG, nitrosoguanidine; Mit C, mitomycin C.

Mutation. Using the procedure of Adelberg et al. (64), cells were incubated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) at 100 µg/ml for 30 min, resulting in the survival of 1.3% of the cells. After growth on a casamino acid medium, auxotrophic mutants were enriched using the D-cycloserine cycling technique (65) and selected by replicating colonies on casamino acid plates onto minimal acetate plates. Amino acid requirements were determined by an auxanographic method.

Curing with mitomycin C. Curing of plasmids was done using the method of Chakrabarty (66). Cells were grown for 2 days in peptone medium containing various amounts of mitomycin C (1 to 30 µg/ml). A culture growing somewhat slowly was diluted and spread on peptone plates. Cured clones were selected by replicating the colonies grown on petone plates onto minimal fluoroacetate or chloroacetate plates. Ability to assimilate the haloacetates was also confirmed by liquid culture.

Conjugation. Conjugation was done by the method of Williams et al. (67). Donor cells were grown in a fluoroacetate medium for 18 hr, and recipient cells in a peptone medium for 5 hr. Equal volumes of donor and recipient cultures were mixed and incubated at 30°C for 2 hr without shaking. The cells were harvested by centrifugation at 1500 x g for 10 min, washed, and resuspended in the original volume of the minimal medium free of any carbon source. Appropriate dilutions were spread on selection plates. For the control, individual suspensions of donor and recipient cells were separately spread on selection plates.

Preparation of cell-free extracts and DEAE-cellulose column chromatography of the dehalogenases. Procedures were as described in the previous section.

Assay of haloacetate dehalogenase. The activities of H-1 and H-2 were assayed by the determination of glycolate produced from fluoroacetate or chloroacetate, as described in the previous section.

Extraction of plasmid DNA. Plasmid was extracted using the method of Hansen and Olsen (68). Cells were suspended in 25% sucrose solution and lysed by the addition of 0.05 M ethylenediaminetetraacetate (EDTA) and 4% sodium dodecyl sulphate (SDS), with several heat pulses at 55°C. The clear viscous lysate thus obtained was subjected to alkaline denaturation, followed by salting out with NaCl to remove chromosome-membrane complexes. Plasmid DNA was concentrated with polyethyleneglycol 6000 and dissolved in a small volume of TES buffer consisting of 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, and 50 mM NaCl.

Agarose gel electrophoresis. Electrophoresis was performed using the method of Meyers et al. (69). Plasmid DNA was mixed with a one-fifth volume of tracking-dye solution (0.07% bromophenol blue, 7% SDS, and 33% glycerol) and electrophoresed in a vertical slab gel (14 x 12 x 0.2 cm) of 0.75% agarose in Tris-borate buffer (89 mM Tris base, 2.5 mM Na_2EDTA, and 89 mM boric acid, pH 8.2) at 100 V for 3.5 hr at room temperature. A gel was stained with 1 µg/ml ethidium bromide solution for 30 min. DNA bands were visualized over an ultraviolet transilluminator and photographed on Kodak 2475 recording film, using orange and UV filters.

RESULTS

Plasmid in Moraxella sp. B

Plasmid DNA was extracted from Moraxella cells grown on peptone and electrophoresed on the agarose gel. It gave two DNA bands; one for plasmid cc DNA, and the other for chromosomal DNA (Fig. 1, lane 1) The plasmid band migrated more slowly than the chromosomal DNA band, and ocDNA stayed at the bottom of a sample hole, suggesting that the plasmid has the larger molecular size.

Curing of dehalogenating activity

To see if the plasmid found is involved in the dehalogenating ability of Moraxella sp. B or in its multiple drug resistances, curing experiments were done using mitomycin C. Cells treated with mitomycin C were grown on peptone plates, and the 574 resulting colonies were replicated onto fluoroacetate and chloroacetate minimal plates. Eight colonies grew well on fluoroacetate but very poorly on chloroacetate (Type I), and 2 colonies were unable to grow on both halo-

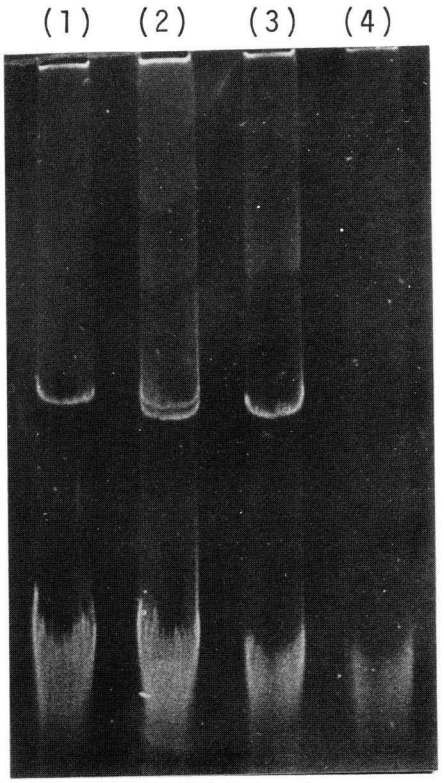


Fig. 1. Agarose Gel Electrophoresis of Plasmid DNA Isolated from Parent and Cured Strains of Moraxella sp. B.

(1) Parent strain B; (2) B + B86; (3) cured strain B86 (Type I); (4) cured strain B123 (Type II). Samples (30 ul) were electrophoresed on 0.75% agarose gel for 3 hr at 100 V. Migration was from top (cathode) to bottom (anode).

acetates (Type II). DEAE-cellulose column chromatography of cell-free extracts revealed that Type I colonies had lost dehalogenase H-2, while Type II colonies had lost both H-1 and H-2.

Upon examining the drug resistance of the cured strains, I saw that Type I strains were resistant to mercuric chloride and all of the antibiotics listed in Table II, as was the parent strain, while Type II strains were susceptible to mercuric chloride. The ability to assimilate glycolate and penicillin G had not altered. Properties of the parent and cured strains are shown in Table II.

From the cured strains, plasmid DNA was extracted and

TABLE II. Properties of Parent and Cured Strains of Moraxella sp. B

Properties	Parent strain	Cured strain	
		Type I	Type II
Assimilation			
Fluoroacetate	+	+	-
Chloroacetate	+	±	-
Glycolate	+	+	+
Penicillin G	+	+	+
Dehalogenase			
H-1	+	+	-
H-2	+	-	-
Resistance			
$HgCl_2$ (10 μg/ml)	+	+	-
Kanamycin (10^3 μg/ml)	+	+	+
Penicillin G (10^3 μg/ml)	+	+	+
Streptomycin (10^4 μg/ml)	+	+	+
Gentamicin (100 μg/ml)	+	+	+
Rifampicin (500 μg/ml)	+	+	+
Nalidixic acid (100 μg/ml)	+	+	+
Curing frequency		8/574	2/574

electrophoresed on an agarose gel (Fig. 1). Type I strains all gave single identical plasmid bands migrating somewhat faster than the parental plasmid (lane 3, for the representative strain B86). The difference between the parental and B86 plasmids was obvious when the two plasmids were mixed and electrophoresed (lane 2); the results seen in Fig. 1 indicate that the Type I plasmid has shortened. Type II strains gave no plasmid band (lane 4, for the representative strain B123). The coincidental disappearance of a plasmid, the dehalogenases H-1 and H-2, and mercury resistance suggests that the plasmid specifies these phenotypes. This plasmid was designated pUO1, and the plasmid of the strain deficient in H-2 (Type I), which seems to be a deletion derivative of pUO1, was designated pUO11.

Spontaneous loss of the H-2 enzyme

When the wild strain was grown in a peptone medium without mitomycin C, H-2 deficient mutants appeared at almost the same frequency as after the mitomycin C treatment. The plasmid of the spontaneous mutant was identical with the plasmid of the Type I cured strain, pUO11. Therefore, the occurrence of Type I cells in the curing experiment was probably not caused by mitomycin C.

Conjugational transfer of plasmid

Strain B86, an auxotrophic (Ile^-, Leu^-, Val^-) mutant of Moraxella B, was obtained by NTG mutagenesis. Using this mutant as the donor and the cured strain B123 as the recipient, conjugational transfer of plasmid pUO1 was attempted. After 2 hr of mating, exconjugants were selected on fluoroacetate minimal plates. The donor and recipient cells failed to give colonies when plated separately on selective

plates. Exconjugant colonies appeared at a frequency of about 10^{-4} per donor cell, and it was found that they could produce both dehalogenases, H-1 and H-2, and that they had become resistant to mercury. Electrophoresis of plasmids showed that the exconjugants contained an identical plasmid with pUO1 of the donor B186 (Fig. 2, for the representative exconjugant P2).

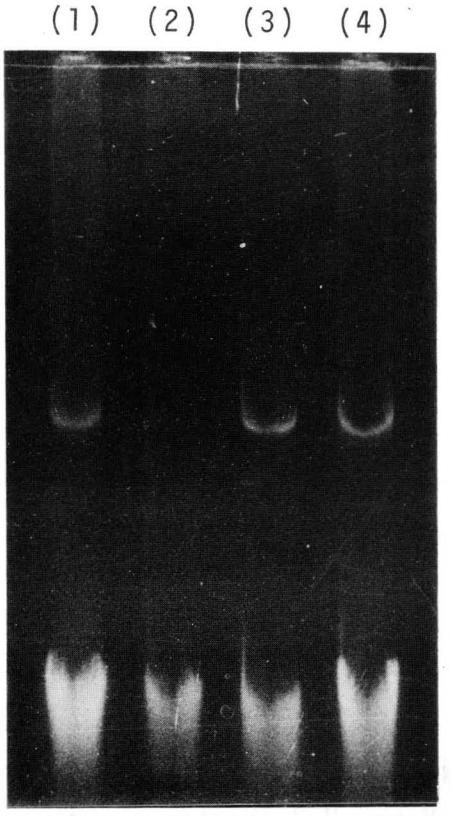


Fig. 2. Agarose Gel Electrophoresis of Plasmid DNA Isolated from Donor B186, Recipient B123 and Exconjugant P2.

(1) B186; (2) B123; (3) P2; (4) B186 + P2. Conditions of electrophoresis were the same as in Fig. 1, except for 3.5-hr running.

In the same way, B44, a mutant of B186 deficient in H-2, was mated with B123. Exconjugants appeared at a frequency of 10^{-4} per donor, and were found to express H-1 and mercury resistance, and also to carry plasmid pUO11. These results are evidence that plasmid pUO1 mediates dehalogenases H-1 and H-2 and mercury resistance, and its deletion plasmid, pUO11, mediates H-1 and mercury resistance.

In addition, plasmid pUO1 was conjugatively transferred from B186 to E. coli K12, Pseudomonas acidovorans, and a glycolate-assimilating isolate of Pseudomonas sp., and was found to express H-1, H-2, and mercury resistance in these hosts.

DISCUSSION

Moraxella sp. B harbors a plasmid that is involved in its assimilation of haloacetate. The plasmid, named pUO1, specified two haloacetate dehalogenases, H-1 and H-2, and mercury resistance. This was the first demonstration of plasmid-dependent dehalogenation in microorganisms.

One microorganism containing two analogous dehalogenases has not been found before now, and it is of further interest that the two analogous enzymes are encoded together on one plasmid. The H-1 enzyme is inducible and proficient in defluorination, while the H-2 enzyme is constitutive and proficient in dechlorination. However, Moraxella cells spontaneously lost the H-2 function. The H-2 gene seems to be deleted from pUO1 easily, resulting in the deletion plasmid, pUO11. Although the H-1 enzyme acts somewhat on chloroacetate, the mutant deficient in H-2 cannot utilize the compound, because the H-1 enzyme is not induced by chloroacetate. Therefore, the H-2 gene is necessary for Moraxella

sp. B to utilize chloroacetate.

Plasmid pUO1 specifies only the first step of the metabolism of haloacetate, namely, dehalogenation of haloacetate, because a plasmid-cured strain can assimilate glycolate. The main function of this plasmid may be to detoxify haloacetate.

Among the many resistances of Moraxella sp. B, mercury resistance alone was dependent upon pUO1. Mercury resistance in many bacteria is determined by plasmids (70), and in some cases, the determinant is carried on a transposon such as Tn501. Plasmid pUO1 is a new mercury-resistant plasmid, but it is not known if the resistance determinant is identical with those of other plasmids and transposons.

SUMMARY

It was found that Moraxella sp. B harbored a plasmid, pUO1. When the plasmid was eliminated by curing with mitomycin C, two haloacetate dehalogenases, H-1 and H-2, and mercury resistance disappeared in the organism. When the plasmid was conjugatively transferred to the cured cells or to Pseudomonas acidovorans, it conferred the H-1 and H-2 enzymes and mercury resistance on the hosts. These observations indicate that the H-1 and H-2 enzymes and mercury resistance are determined by the plasmid. A spontaneous mutant deficient in H-2 appeared frequently, and it harbored a shortened plasmid, pUO11, which specified the H-1 enzyme and mercury resistance.

Section 2

Isolation and Properties of Plasmid pUO1 that Determines Two Haloacetate Dehalogenases and Mercury Resistance in Moraxella sp. B

In the previous section, it was reported that Moraxella sp. B harbors a plasmid, pUO1, that determines two haloacetate dehalogenases, H-1 and H-2, and mercury resistance. The H-2 function, however, was spontaneously lost at the frequency of a few percents, and such a mutant deficient in H-2 harbored a plasmid rather smaller than pUO1, which was found to confer the H-1 enzyme and mercury resistance on the host. The smaller plasmid, pUO11, seemed to be a deletion derivative of pUO1. In this section, the purification and characterization of pUO1 and pUO11 are described; pUO11 is confirmed to be a deletion mutant of pUO1, and the deletion size is estimated by comparing the molecular sizes of pUO1 and pUO11.

MATERIALS AND METHODS

Bacterial strains and plasmids. Moraxella sp. B (pUO1) and its derivative B86 (pUO11) have been described previously. Plasmids used as molecular size standards and their hosts are listed in Table I.

Media and cultural conditions. Moraxella sp. B and B86 and Pseudomonas aeruginosa PAO303 were aerobically grown for 24 hr at 30°C in a medium containing 1% Polypepton, 0.16% KH_2PO_4, 0.65% $Na_2HPO_4.12H_2O$, 0.1% NH_4NO_3, 0.02% $MgSO_4.7H_2O$, and 0.01% yeast extract, pH 7.0. Strains of E. coli was aerobically grown overnight at 37°C in the medium containing

TABLE I. Plasmids Used as Molecular Size Standards

Plasmid	Molecular size (Mdal)	Host	Reference
ColE1	4.2	E. coli K12 A745	72
pMG1'	20	Ps. aeruginosa PAO303	68
PVA517A	35.8	E. coli V517	73
RP4	36	E. coli 20SO	74
F	63	E. coli K12 Y mel	75
pTN2	74	E. coli 20SO	76

1% Polypepton, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose, pH 7.0. To amplify plasmid ColE1, 0.01% chloramphenicol was added to a culture of E. coli K12 A745 when the growth reached the OD_{610} of 0.4, and incubation was continued overnight.

Extraction of plasmid DNA. Extraction of plasmid DNA from Moraxella and Pseudomonas cells was done by the method of Hansen and Olsen (68), and from E. coli cells by the cleared-lysate method of Clewell and Helinski (77). Plasmids pUO1 and pUO11 were isolated separately in the following way.

Cells harvested from 3.5 liters of culture were washed in 0.05 M Tris-HCl buffer (pH 8.0), and resuspended in 270 ml of 25% sucrose-0.05 M Tris-HCl buffer (pH 8.0). A 20-ml portion of lysozyme solution (10 mg/ml) and 100 ml of 0.25 M EDTA were added. Lysis was done by adding 100 ml of 20% SDS, followed by eight cycles of a 55°C heat pulse and mixing. The cleared viscous solution thus obtained was denatured at pH 12.2 for 3 min, and after neutralization with 2 M Tris-HCl (pH 7.0), 20% SDS and 5 M NaCl were added to the final concentrations of 4% and 1 M, respectively, and the mixture

was left overnight at 4°C. The white floc that formed was removed by centrifugation, and to the supernatant fluid, polyethyleneglycol 6000 was added to a final concentration of 10%. The precipitate was collected by centrifugation and dissolved in 20 ml of TES buffer (0.05 M Tris-HCl, 5 mM EDTA, and 0.05 M NaCl, pH 8.0).

Phenol extraction. To 20 ml of the crude plasmid solution, the same volume of TES-saturated phenol was added and mixed gently. After centrifugation, the aqueous phase was washed with ether several times, mixed with two volumes of cold ethanol, and kept at -20°C for more than 2 hr. The precipitated DNA was collected by centrifugation at 7,000 x g for 10 min, dissolved in 4 ml of TES buffer, and dialyzed overnight against the same buffer.

CsCl-ethidium bromide (EtBr) equilibrium density gradient centrifugation. Plasmid DNA was further purified by CsCl-EtBr equilibrium centrifugation, as described by Radoloff et al. (78). To 4.5 ml of DNA solution, 4.5 g of solid CsCl and 0.25 ml of 1% EtBr were added, and the refractive index was adjusted to 1.392. Centrifugation was at 40,000 rpm at 15°C for 40 hr in a Hitachi RP-65 angle rotor. After this run, a satellite (lower) band was collected as one fraction by puncturing the bottom of the polyallomer tube. The fraction was extracted 5 times with cold isopropanol to remove ethidium bromide, and dialyzed against cold TES buffer.

Agarose gel electrophoresis. Electrophoresis conditions were as described in the previous section. For estimating the molecular sizes of plasmids, a 0.65% agarose gel and Tris-borate buffer were used. For analyzing restriction

fragments of plasmid DNA, electrophoresis was done in a 1% agarose gel in E buffer (40 mM Tris-acetate, 20 mM sodium acetate, and 5 mM EDTA, pH 8.2; 79) at a constant current of 10 mA for 18 hr. The sizes of the DNA fragments were estimated using phage λ DNA-HindIII fragments (14.6, 6.13, 4.07, 2.83, 1.45, 1.26, and 0.43 Mdal) as the mobility reference standards.

Electron microscopy. Covalently closed circular (ccc) DNA was converted to open circular (oc) DNA by incubation with an adequate amount (0.01 µg/ml) of deoxyribonuclease I, or by freezing and thawing of the DNA. DNA samples were prepared for electron microscopy using the aqueous basic-protein film technique of Kleinschmidt (80); the spreading solution was 0.5 M ammonium acetate containing 0.01% cytochrome c and ocDNA, and the hypophase was 0.25 M ammonium acetate (pH 7.5). The cytochrome c film was picked up on polyvinyl formvar-coated copper grids, and stained in uranyl acetate. The preparation was shadowed with platinum-palladium (80:20) on a rotary table. The DNA molecules were photographed in a Hitachi H-300 electron microscope.

Contour lengths of ocDNA were measured by projecting electron-micrograph negatives on a screen and by tracing the images on paper; the length was measured using a curvimeter. Molecular weights of plasmids were determined using ColE1 DNA as an internal length standard (4.2 Mdal).

Cleavage of plasmid DNA with restriction endonucleases. Plasmid DNA was dialyzed against TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0), and digested with a restriction enzyme at 37°C (except for SmaI, which was at 30°C) for 4 hr in, normally, 20 µl of reaction mixture containing 0.3 to 0.5

μg of DNA, 4 units of restriction enzyme, and the buffer solution given in Table II.

Materials. Phage λ DNA and the restriction endonucleases EcoRI, SalI, HindIII, BamHI, and SmaI were all purchased from the Takara Shuzo Co. Ltd., Kyoto.

TABLE II. Buffer Solutions Used in Restriction Endonuclease Digestions

Enzyme	Components					
	Tris-HCl (mM)	$MgCl_2$ (mM)	NaCl (mM)	2-ME (mM)	EDTA (mM)	BSA (%)
EcoRI	50 (pH 7.5)	7	100	7	-	0.01
SalI	10 (pH 7.5)	7	175	7	0.2	0.01
HindIII	10 (pH 7.5)	7	60	-	-	-
BamHI	10 (pH 8.0)	7	100	2	-	0.01
SmaI	10 (pH 8.0)	7	20 (KCl)	7	-	0.01

2-ME, 2-mercaptoethanol; BSA, bovine serum albumin.

RESULTS

Purification of pUO1 and pUO11 and agarose gel electrophoresis

Plasmids pUO1 and pUO11 were isolated from Moraxella sp. B and its derivative B86, respectively, and purified by CsCl-EtBr equilibrium centrifugation. The freshly purified plasmids migrated as single well-defined bands on agarose gel electrophoresis, indicating that these preparations contained single species of DNA molecules. However, old preparations, especially one subjected to repeated freezing and thawing, gave another DNA band which stayed at the bottom of the gel slot on electrophoresis. These observations suggest that

the purified pUO1 and pUO11 are cccDNA molecules, which are converted into oc form by repeated freezing and thawing.

The molecular sizes of pUO1 and pUO11 were estimated to be about 43 Mdal and 39 Mdal, respectively, by measuring the relative mobilities against reference plasmids (Fig. 1).

Electron microscopy of pUO1 and pUO11

Purified plasmids were converted to the oc form and observed under an electron microscope. Figure 2 is an electron micrograph of an ocDNA molecule of pUO1. The pUO11 molecules were also observed in oc form (photograph not shown). Molecular weights of pUO1 and pUO11 were determined

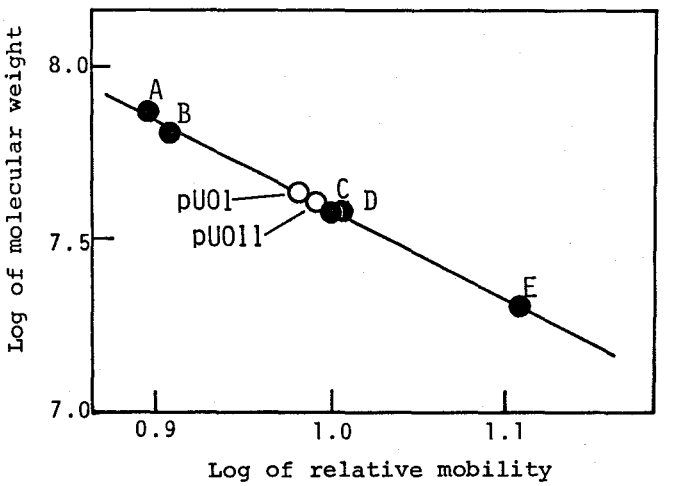


Fig. 1. Determination of Molecular Weights of pUO1 and pUO11 by Agarose Gel Electrophoresis.

Purified plasmid DNA was electrophoresed on a 0.65% agarose gel in Tris-borate buffer at 25 mA for 3.5 hr. Mobility reference plasmids: A, pTN2 (74 Mdal); B, F (63 Mdal); C, pVA517A (35.8 Mdal); D, RP4 (36 Mdal); E, a small plasmid coexisting with pMG1 (20 Mdal). Plasmid RP4 was set arbitrarily at mobility 10.

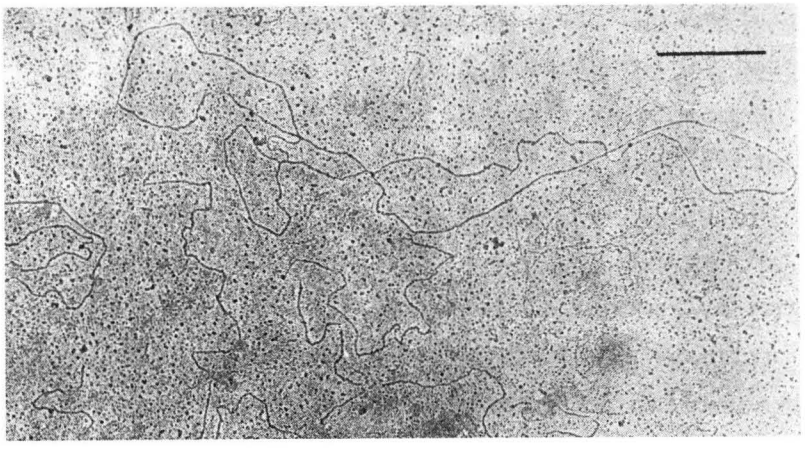


Fig. 2. Electron Micrograph of an Open Circular DNA Molecule of pUO1.

The sample was prepared using Kleinschmidt technique. The bar represents 1 μm.

by measuring the contour lengths of ocDNA molecules. The histograms of contour lengths are presented in Fig. 3. Using a conversion factor calculated from the contour length of an internal standard ColE1 DNA (4.2 Mdal), the molecular weights of pUO1 and pUO11 were estimated to be 43.7 ± 1.6 Mdal and 40.1 ± 1.3 Mdal, respectively.

Cleavage analysis with restriction endonucleases

To compare the DNA molecules of pUO1 and pUO11, they were digested by EcoRI, SalI, BamHI, HindIII, or SmaI. The electrophoretic patterns of these digests were very similar between pUO1 and pUO11, except that one or two fragment bands were missing in the pUO11 digests (Fig. 4). This similarity indicates that pUO11 is a deletion mutant derived from pUO1.

To make the cleavage patterns more immediately under-

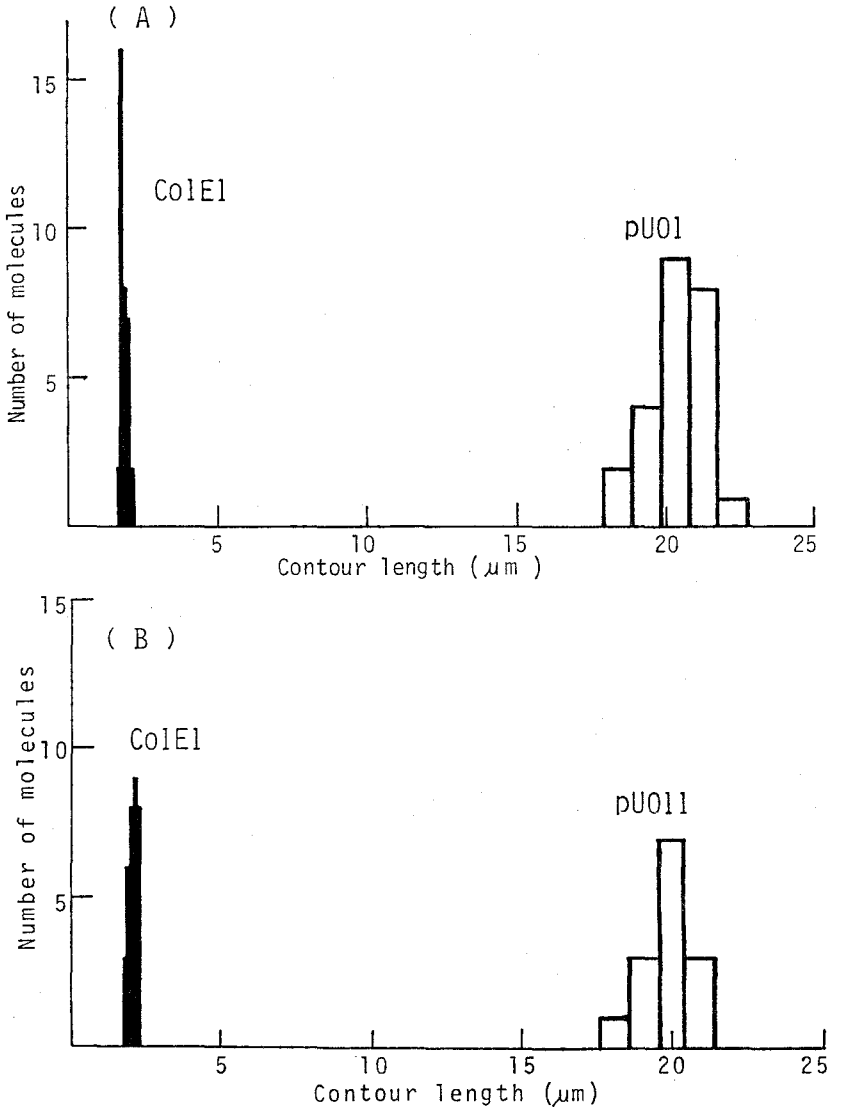


Fig. 3. Histograms of Contour Lengths of Open Circular DNA Molecules of pUO1 (A) and pUO11 (B).

ColE1 DNA (4.2 Mdal) was used as an internal standard. The conversion factors were 2.14 Mdal per μm in (A) and 2.02 Mdal per μm in (B).

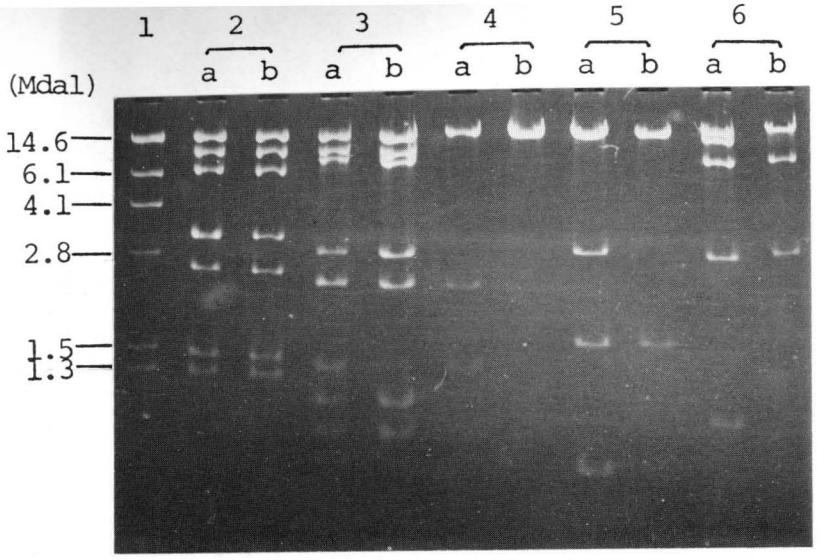


Fig. 4. Electrophoretic Patterns of pUO1 and pUO11 Digested with Various Restriction Endonucleases.

1, phage λ DNA-HindIII fragments; 2, EcoRI digests; 3, SalI digests; 4, BamHI digests; 5, HindIII digests; 6, SmaI digests. The digests of pUO1 (a) and pUO11 (b) were electrophoresed on a 1% agarose gel in E buffer at 10 mA for 10 hr.

TABLE III. Sizes of DNA Fragments of pUO1 and pUO11 Digested by Restriction Endonucleases

The sizes (in Mdal) were calculated from data given in Fig. 4 and additional experiments. The sums of fragment sizes are shown in parentheses.

EcoRI		SalI		HindIII		BamHI		SmaI	
pUO1	pUO11	pUO1	pUO11	pUO1	pUO11	pUO1	pUO11	pUO1	pUO11
15.0	15.0	14.8	14.8	17.8	17.8	21.9	21.9		29.0
9.6	9.6	9.6	9.6	17.8	17.8	17.8	17.8	17.8	
6.6	6.6	8.2	8.2	3.0		2.2		12.6	
3.1	3.1	2.8	2.8	1.48	1.48	1.22		7.4	7.4
3.0		2.2	2.2	0.60	0.60	(43.1)	(39.7)	2.8	2.8
2.4	2.4	2.2		(40.7)	(37.7)			0.89	
1.35	1.35	1.20						0.50	
1.17	1.17	0.95	0.95					(42.0)	(39.2)
0.50	0.50	0.76	0.76						
(42.7)	(39.7)	(42.7)	(39.3)						

standable, Table III was prepared from the data given in Fig. 4 and from additional experiments. EcoRI digested pUO1 DNA, generating nine fragments; eight seemed identical to fragments of EcoRI-pUO11. SalI-digestion of pUO1 also generated nine fragments, seven of which seemed identical to ones of pUO11.

HindIII-pUO1 and -pUO11 digests had four and three bands, respectively, on electrophoresis. However, top-band DNA (17.8 Mdal) in both cases, which was extracted from the gels using the method of Vogelstein (81), was digested by EcoRI, generating five fragments, and the sum of the sizes was 35.5 Mdal in both cases. This indicates that two species of 17.8-Mdal fragments overlap in the top bands. A BamHI-pUO1 digest generated four fragments; two seemed identical to ones from a pUO11 digest.

SmaI cleaved pUO1 to give six fragments and pUO11 to give three fragments. Two fragments of pUO11 seemed identical to two of pUO1, but one was a new large fragment, whose size was close to the sum of the two other pUO1 fragments, 17.8 and 12.6 Mdal. Probably the formation of the large fragment is due to the disappearance of the cleavage sites located in the deleted region. Sums of the fragment sizes are also given in Table III.

Molecular sizes of pUO1 and pUO11 and deletion size

The sums of the fragment sizes are in agreement with those calculated from the contour lengths of oc DNA and the relative mobilities of cccDNA. Molecular sizes estimated by these three methods are summarized in Table IV.

Averaging the differences between the molecular sizes of pUO1 and pUO11, I concluded that the size of the deleted DNA segment was about 3.6 Mdal (5.8 kb).

TABLE IV. Molecular Sizes of pUO1 and pUO11

	Molecular sizes (Mdal) determined by			
	Migration of ccc DNA	Contour length	Sum of fragment sizes	
			EcoRI	SalI
pUO1	43	43.7 ± 1.6	42.7	42.7
pUO11	39	40.1 ± 1.3	39.7	39.3
Δ	4	3.6	3.0	3.4

DISCUSSION

Agarose gel electrophoresis showed that the plasmids pUO1 and pUO11 are of cccDNA, which is readily converted to ocDNA by rough handling. This was confirmed by electron microscopy; supercoiled DNA molecules were seen together with relaxed ocDNA. The digestion patterns of pUO1 and pUO11 closely resembled each other, except that one or two fragments were missing in pUO11, so pUO11 is a deletion mutant derived from pUO1.

In general, a deletion mutant should be missing one or more restriction fragments and should generate one new fragment. When plasmid pUO11 was digested with SmaI, it lost four fragments and generated a new large fragment. However, digested with other restriction enzymes, generation of a new fragment was not observed, although one or two fragments were missing in every case. Perhaps new fragments that must be in fact generated from pUO11 would be very similar to the original fragments of pUO1. Larger fragments did not have electrophoretic mobilities different enough for them to be distinguished, and their sizes would have more than the usual amount of deviation. Therefore, the digests without larger fragments found here, such as the EcoRI or SalI

digests, were used for calculating the plasmid size.

Molecular sizes as estimated by three methods were in agreement with each other. The values 43.7 and 40 Mdal, calculated from the contour lengths, were adopted as the molecular sizes of pUO1 and pUO11, respectively. Therefore, the size of the deletion was estimated to be 3.6 Mdal. That the H-2 enzyme gene is located on the deletion region seems reasonable, since 3.6 Mdal is large enough to encode the H-2 enzyme, with a molecular weight of 43,000.

SUMMARY

Plasmids pUO1 and pUO11 were purified by CsCl-EtBr equilibrium centrifugation, and analyzed by agarose gel electrophoresis, electron microscopy, and digestion with restriction enzymes. The molecular sizes of the plasmids were determined by measuring contour lengths of ocDNA; pUO1 was 43.7 Mdal and pUO11 was 40.1 Mdal. That the restriction patterns of pUO1 and pUO11 were similar indicated that pUO11 is a deletion derivative of pUO1. The size of the deletion was estimated to be 3.6 Mdal.

Section 3
Cleavage Maps of Plasmid pUO1 and Its Deletion Derivative pUO11

Plasmid pUO1 encodes the genes for dehalogenases H-1 and H-2 and mercury resistance. pUO1 frequently loses the H-2 function coincidently with the loss of a specific segment of about 3 Mdal. To estimate the loci of these genes and of the deleted region, and to study the fine structure of pUO1 and the functions of the genes, it is necessary to make a restriction map of pUO1. This section describes the mapping of cleavage sites for five restriction endonucleases, BamHI, HindIII, SmaI, EcoRI, and SalI, and the deleted region is localized.

MATERIALS AND METHODS

Organisms and plasmids. Plasmids pUO1 and pUO11 were prepared from Moraxella sp. B and its deletion mutant B86, respectively. The organisms were grown as described in the previous section.

Purification of plasmids. Plasmid DNA was extracted by the method of Hansen and Olsen (68) and purified by CsCl-EtBr density gradient centrifugation as previously described.

Cleavage of plasmid DNA with restriction endonucleases. The restriction endonucleases used were purchased from Takara Shuzo Co., Ltd., Kyoto, and DNA digestion with them was done under the conditions recommended by the supplier.

For double digestions involving enzymes with different

buffer requirements, DNA was first digested with the enzyme requiring the buffer of lower ionic strength, and then the buffer was adjusted with concentrated components for the second enzyme. Another kind of double digestion was done as follows. The first digest products were separated by agarose gel electrophoresis and recovered by electroelution (82) from bands cut from the stained gel, and then individual DNA fragments were digested with the second enzyme.

Partial digestion was achieved by limiting the enzyme quantity, the reaction period, or both.

Agarose gel electrophoresis. Digested DNA products were analyzed by electrophoresis in 0.8% or 1% agarose vertical slab gels in the manner described previously. Fragments of phage λ DNA digested with HindIII or EcoRI were used as molecular weight standards (83).

RESULTS

Restriction fragments of pUO1 and pUO11

The fragments generated from pUO1 and pUO11 digested by EcoRI, SalI, BamHI, SmaI, and HindIII have already been described. The names (alphabetic letters) and sizes of these fragments are listed in Table I. The pUO1 DNA yielded nine fragments on digestion with EcoRI and with SalI, and four, five, and six fragments with BamHI, HindIII, and SmaI, respectively. The pUO11 DNA yielded two, three, and four fragments on digestion with BamHI, SmaI, and HindIII, respectively. Therefore, the first step in constructing a map of pUO1 was to create a pUO11 map for BamHI, HindIII, and SmaI, and the next step was to supplement the deleted region. The final step was to add the cleavage sites for EcoRI and

TABLE I. Restriction Fragments of pUO1 and pUO11

Fragment	Size (Mdal) of fragment generated by digestion with									
	BamHI		HindIII		SmaI		EcoRI		SalI	
	pUO1	pUO11	pUO1	pUO11	pUO1	pUO11	pUO1	pUO11	pUO1	pUO11
A,A'	21.9	21.9*	17.8	17.8*	17.8	29.0*	15.0	15.0	14.8	14.8
B	17.8	17.8	17.8	17.8	12.6		9.6	9.6	9.6	9.6
C,C'	2.2		3.0		7.4	7.4	6.6	6.6	8.2	8.2*
D	1.22		1.48	1.48	2.8	2.8	3.1	3.1	2.8	2.8
E			0.60	0.60	0.89		3.0		2.2	2.2
F					0.50		2.4	2.4	2.2	
G,G'							1.35	1.35*	1.20	
H							1.17	1.17	0.95	0.95
I							0.50	0.50	0.76	0.76

* New fragment, not necessarily identical in the DNA composition to the counterpart of pUO1, and distinguished by putting a prime on the alphabetical names.

SalI. Mapping was done by analysis of size-distribution patterns of DNA fragments generated by double and partial digestions with restriction endonucleases.

Mapping of BamHI, SmaI, and HindIII cleavage sites in pUO11

The relative order on the DNA molecule of fragments of pUO11-BamHI (2 pieces) and of pUO11-SmaI (3 pieces) were examined by analysis of overlapping sets of fragments, using data from double digestions involving redigestion of individual fragments recovered from the gel (Table II). Fragment C (6.5 Mdal) from the SmaI-BamHI double digestion was produced from the BamHI-fragment B (BamB) and also from the SmaI-fragment C (SmaC); i. e., BamB overlaps SmaC by a length corresponding to 6.5 Mdal. Similarly, fragment B (11.2 Mdal) from SmaI-BamHI digestion was produced from BamB and necessarily from SmaA'; i. e., BamB also overlaps SmaA' by a length corresponding to 11.2 Mdal. This reasoning suggests the relative positions of the BamHI and SmaI sites to be as shown in Fig. 1a.

TABLE II. Fragments Generated by Double Digestion of pUO11 and pUO1 with BamHI, HindIII and SmaI

The fragments in brackets were recovered from gels and redigested with the second enzyme.

Enzyme	Size (Mdal) of fragment										
	A	B	C	D	E	F	G	H	I	J	K
pUO11											
SmaI-BamHI	16.8	11.2	6.5	2.8	0.71						
[SmaC]-BamHI			6.5		0.71						
[BamB]-SmaI		11.2	6.5								
SmaI-HindIII	16.8	10.4	7.4	1.48	0.99	0.60	ND				
[SmaD]-HindIII				1.48	0.99		ND				
[HinA'&B]-SmaI	16.8	10.4	7.4		0.99		ND				
HindIII-BamHI	17.8	16.8	1.48	0.83	0.60	ND					
[HinA'&B]-BamHI	17.8	16.8		0.83		ND					
pUO1											
SmaI-BamHI	16.8	11.2	6.5	2.8	1.07*	0.89*	0.87*	0.71	0.35*	ND	
[BamB]-SmaI		11.2	6.5								
[BamC]-SmaI						0.89	0.87		0.35		
[BamD]-SmaI					1.07					ND	
[SmaA]-BamHI	16.8				1.07						
[SmaB]-BamHI		11.2					0.87				
SmaI-HindIII	16.8	10.4	7.4	1.48	1.05*	0.99	0.89*	0.60	0.50*	ND	ND
[HinC]-SmaI					1.05		0.89		0.50		ND
HindIII-BamHI	17.8	16.8	1.48	1.48*	1.22*	0.83	0.60	ND	ND		
[HinC]-BamHI				1.48	1.22			ND			

* Fragments missing in pUO11 digests.

ND, fragment not detected, but its presence can be predicted.

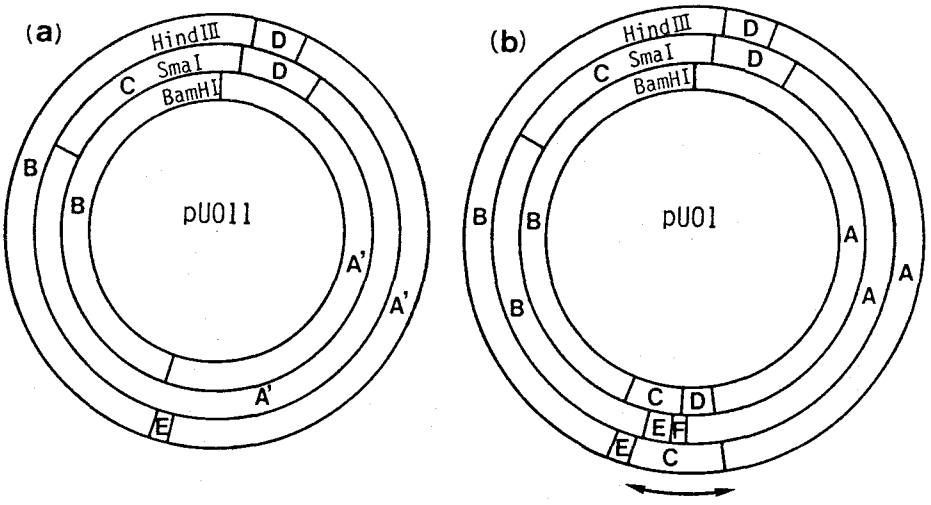


Fig. 1. Cleavage Maps of pUO11 (a) and pUO1 (b) for BamHI, SmaI and HindIII.

Alphabetical letters represent the fragment names given in Table I. An arrow outside the circle indicates the region deleted in pUO11.

Next, the order of HindIII-pUO11 products was examined. Since HinA' and HinB were not distinguishable, there were two possible orders for the HindIII fragments (4 pieces), with HinD either adjacent to HinE or not. It can be seen from the data on the HindIII-SmaI double digestion in Table I that HinD (1.48 Mdal) is entirely included within SmaD, and that HinE (0.60 Mdal) is within SmaA' because the other fragments, SmaC and D, did not produce HinE. This means that HinD is not flanked by HinE, so that the order of HindIII fragments is probably A'-E-B-D (B-E-A'-D is equivalent). The relationship between the maps of HindIII sites and of the BamHI-SmaI sites was established by similar analysis of overlapping sets of fragments, and the results are shown in Fig. 1a.

Cleavage sites of BamHI, SmaI, and HindIII in the deletion region

Fragments originating from the deletion region were analyzed to estimate their relative positions. In general, a deletion mutant is to generate one new fragment in which a junction point of DNA left after deletion is present. One large fragment, SmaA', of pUO11 was a new fragment composed of DNA left over from two pUO1-fragments, SmaA and SmaB, after deletion. Therefore, the junction point was probably near the center of SmaA'. The pUO1-fragments SmaE and SmaF, both generated from the deletion region, must be between SmaA and SmaB.

Analysis of the products of SmaI-BamHI double digestion revealed that SmaA overlaps BamA and BamD by lengths corresponding to 16.8 and 1.07 Mdal, respectively, and that SmaB overlaps BamB and BamC by 11.2 and 0.87 Mdal, respectively. It was also found that SmaE is flanked by SmaB, and SmaF by SmaA. HinC was easily located in relation to BamC and BamD; it overlaps BamC by 1.48 Mdal, and includes all of BamD. Thus, the cleavage sites of BamHI, SmaI, and HindIII were mapped on pUO1 as shown in Fig. 1b.

Mapping of EcoRI and SalI cleavage sites in pUO1 and pUO11

The order of EcoRI-pUO11 fragments could be roughly estimated by analysis of overlap with the SmaI-generated fragments (Table III) in the following way. Fragments EcoA and EcoG are included in SmaA, and EcoC, EcoF, and EcoH are in SmaB. EcoB overlaps SmaC and SmaB by lengths corresponding to 7.4 and 1.05 Mdal, respectively, and EcoD overlaps SmaD by 2.2 Mdal. EcoE, missing in the pUO11 digests, ought to overlap the deleted region. EcoG must be near SmaF within SmaA because EcoG has a HindIII site (see Fig. 1b).

TABLE III. Fragments Generated by Double Digestion of pUO1 with EcoRI and Other Restriction Enzymes

Enzyme	Size (Mdal) of fragment														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
EcoRI-BamHI	15.0	8.6	6.6	3.1	2.4	2.1*	1.26	1.17	1.12	0.83*	0.48	ND	ND		
[EcoB]-BamHI		8.6					1.26								
[EcoD&E]-BamHI				3.1		2.1				0.83					
[EcoI]-BamHI											0.48		ND		
[BamA]-EcoRI	15.0			3.1			1.26		1.12						
[BamB]-EcoRI		8.6	6.6		2.4			1.17			ND				
EcoRI-HindIII	15.0	9.5	6.6	2.4	2.4*	1.95	1.17	1.12	0.80	0.58	0.58*	0.45	ND	ND	
[EcoD&E]-HindIII					2.4	1.95		1.12		0.58					
[EcoG]-HindIII									0.80		ND				
[EcoI]-HindIII												0.45		ND	
[HinC]-EcoRI					2.4						0.58				
[HinE]-EcoRI										0.58				ND	
EcoRI-SmaI	15.0	7.4	6.6	2.4	2.2	1.35	1.17	1.05	0.89*	0.85*	0.81	0.66*	0.50*	0.50	0.50
[EcoB]-SmaI		7.4						1.05						0.50	
[EcoD&E]-SmaI					2.2				0.89	0.85	0.81	0.66	0.50		
[SmaA]-EcoRI	15.0					1.35				ND	ND				
[SmaB]-EcoRI			6.6	2.4			1.17	1.05				ND			ND
[SmaC]-EcoRI		7.4													
[SmaD]-EcoRI					2.2									ND	

The footnotes are the same as those of TABLE II.

Therefore, the order of EcoRI fragments is probably A-D-B-(C, F, H)-E-G, with the internal order of C, F and H and the position of fragment I remaining unclear.

Analysis of partially digested products of pUO11-EcoRI (Table V) provided evidence that the order of the small fragments is H-F-I-G'. Thus, the order of EcoRI-generated fragments was established to be A-D-B-C-H-F-I-G' for pUO11 and A-D-B-C-H-F-I-E-G for pUO1.

Table III also indicates that EcoRI fragments B, E, G, and I each have one cleavage site for BamHI; that fragments D, E, G, and I have one site for HindIII; and that fragments B, D, and E have two, one, and three sites, respectively, for SmaI. Taking into account this information and the overlapping length of EcoRI fragments with BamHI, SmaI, and HindIII fragments, the position of the EcoRI sites relative to the other restriction sites could be readily estimated; the results are shown in Fig. 2a and b.

In the same way, SalI fragments were mapped using the data in Tables IV and V, and results are shown in Fig. 2a and b. These maps are in conformity with the double digestion data presented in Tables II, III and IV.

DISCUSSION

Cleavage maps of pUO1 and its deletion derivative pUO11 were constructed for five restriction endonucleases, BamHI, SmaI, HindIII, EcoRI, and SalI, which all recognize six base pair sequences. The location of each cleavage site was established by analysis of double- and partial-digest products. However, two cleavage sites less than 0.1 Mdal apart, if present, might be regarded as a single site because no particular efforts were made to detect products smaller than 0.1 Mdal.

TABLE IV. Fragments Generated by Double Digestion of pUO1 with SalI and Other Restriction Enzymes

Enzyme	Size (Mdal) of fragment																	
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
SalI-EcoRI	9.6	9.6	6.5	5.6	1.55	1.51	1.51*	1.35	1.20*	1.17	1.05	0.95	0.76	0.63	0.50	0.50	0.30	ND
[SalA]-EcoRI	9.6			5.6		1.51												
[SalB]-EcoRI		9.6																
[SalC]-EcoRI			6.5					1.35										ND
[SalD]-EcoRI										1.17	1.05				0.50			
[SalE&F]-EcoRI					1.55		1.51							0.63		0.50	0.30	
[EcoD&E]-SalI					1.55	1.51	1.51		1.20									ND
[EcoF]-SalI											1.05	0.95					0.30	
SalI-BamHI	12.3	9.6	7.0	2.8	2.8	2.2	1.51*	0.95	0.76	0.65	0.65*	0.65*	0.56*					
SalI-HindIII	13.3	9.6	6.8	2.8	2.2	1.48	1.20*	0.95	0.89*	0.76	0.65	0.65*	0.60	ND				
SalI-SmaI	9.6	8.2	7.4	6.3	2.8	2.1	1.70*	1.51	0.95	0.76	0.68	0.56*	0.50*	0.40*	ND			

The footnotes are the same as those of TABLE II.

TABLE V. Partial Digestion with EcoRI and SalI

	Partial digestion products (Mdal)	Possible constituents (size in Mdal)
	4.2	F (2.4) + G' (1.35) + I (0.50)
	4.0	F (2.4) + H (1.17) + I (0.50)
pUO11-EcoRI	3.6	F (2.4) + H (1.17)
	2.9	F (2.4) + I (0.50)
	1.9	G' (1.35) + I (0.50)
	4.6	D (2.8) + H (0.95) + I (0.76)
pUO1-SalI	3.8	D (2.8) + H (0.95)
	3.4	E or F (2.2) + G (1.20)

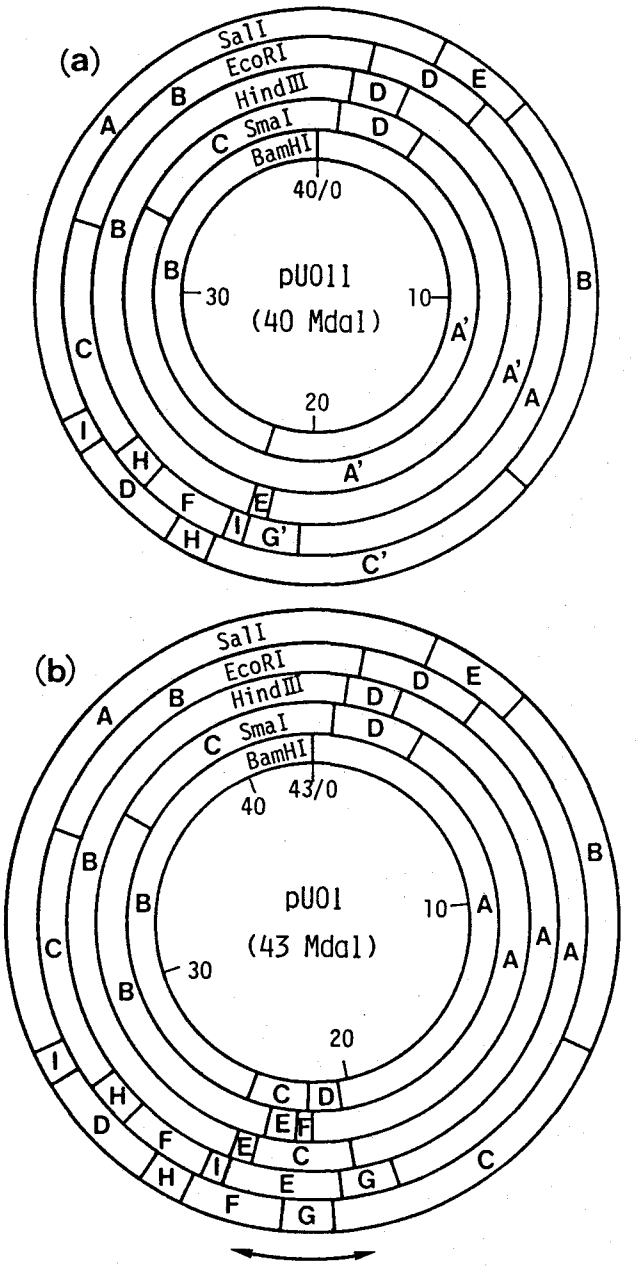


Fig. 2. Cleavage Maps of pUO11 (a) and pUO1 (b) for BamHI, SmaI, HindIII, EcoRI and SalI.

The BamHI site between the fragments BamA and BamB is used as a reference point. The map is calibrated in Mdal. An arrow outside the circle indicates the region deleted in pUO11.

In general, there should be one fragment that is only found with the deletion mutant. However, in the pUO11 digests with BamHI, HindIII, EcoRI, and SalI, no fragments were found that were not in the pUO1 digests (Table I). This suggests two possibilities: (i) the new fragment is too small to be detected or (ii) the change in fragment size is too small to be distinguished from the counterpart pUO1 fragment. The map constructed supports the second possibility.

It can be seen from this map that the distribution of cleavage sites is possibly non-random and that most of the sites are crowded in the lower region of the 10-Mdal span. This region includes the readily deleted 3-Mdal segment, which perhaps carries the H-2 gene. The mechanism of this deletion is unknown, but some special DNA structure, such as repeated sequences, may be involved in the deletion. Using the map of pUO1, I will discuss the fine structure of the plasmid DNA and the locations of H-1 and H-2 genes in later sections.

SUMMARY

Restriction maps of pUO1 and pUO11 for BamHI, SmaI, HindIII, EcoRI, and SalI were constructed by analysis of double- and partial-digestion products. By comparing the two maps, the locus of the deleted region missing in pUO11 was estimated on the pUO1 map.

Section 4
Plasmid pUO2 Harbored by Pseudomonas sp. C

I mentioned in Chapter I that a fluoroacetate-assimilable strain, Pseudomonas sp. C, possesses two haloacetate dehalogenases that are very similar to the Moraxella enzymes, H-1 and H-2, in substrate specificities and chromatographic profiles, and so I called the Pseudomonas enzymes H-1 and H-2. Also, one difference, however, is that Moraxella B inducibly produces H-1 and constitutively produces H-2, while Pseudomonas C produces both enzymes constitutively. In addition, Pseudomonas C produces H-2 in larger amounts than does Moraxella B. A preliminary observation was that Pseudomonas C harbored a plasmid about the same size as that of pUO1. Here, discussion senters on whether the dehalogenases of Pseudomonas C are specified by the plasmid, and if they are, whether the plasmid is analogous to pUO1 or not.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Pseudomonas sp. C and its culture conditions were described in Chapter I. Strains B, B86, and B123 of Moraxella sp., and plasmids pUO1 and pUO11 were described in Section 1 of this chapter. Plasmid ColE1 was used as an internal standard during electron microscopic observation.

Assay of haloacetate dehalogenase. The assay system was described in Chapter I. Amounts of H-1 and of H-2 in a cell extract were determined without their being separated from each other; the activity of the cell extract against fluoroacetate was taken to be the amount of H-1, and the

activity against chloroacetate, from which one-fifth of the activity against fluoroacetate was subtracted, was taken to be the amount of H-2.

Curing. Curing of plasmid with mitomycin C or acridine orange was done using the method described in Section 1 of this chapter.

Conjugation. Using the centrifugation method of Stuy (84), mating was carried out in the following way. Donor cells were grown for 24 h in chloroacetate medium; recipient cells were grown for 20 h in peptone medium, then 4 h in fresh medium. Two milliliters of both the donor and the recipient cultures were mixed and centrifuged at 3,000 rpm for 5 min. The cell pellet was washed in fresh peptone medium, and then incubated at 30°C for 2 h. The cells were resuspended in 4 ml of a medium containing no carbon source, and appropriate dilutions were plated on selection medium.

Analyses of plasmid. The procedures used for the extraction and purification of plasmid DNA, CsCl-EtBr equilibrium ultracentrifugation, electron microscopy, restriction endonuclease digestion, and agarose gel electrophoresis were described in Section 2.

RESULTS

A plasmid in Pseudomonas sp. C

Plasmid DNA was extracted from peptone-grown Pseudomonas cells using the method of Hansen and Olsen (68), and electrophoresed on an agarose gel. The sample gave a single plasmid band whose mobility was not distinguishable from that of

Moraxella plasmid pUO1 (43.7 Mdal) (Fig. 1, Lanes 1, 2, and 3).

On ultracentrifugation of the crude plasmid in a CsCl-EtBr buoyant density gradient, a satellite DNA band was observed. It was confirmed by electron microscopic observation that the satellite DNA was supercoiled. The contour lengths of open circular DNA molecules of the plasmid were measured, and the size of the plasmid was estimated to be 43 Mdal, using ColE1 DNA as an internal standard. This value is very close to that of pUO1. The plasmid of Pseudomonas C was designated pUO2.

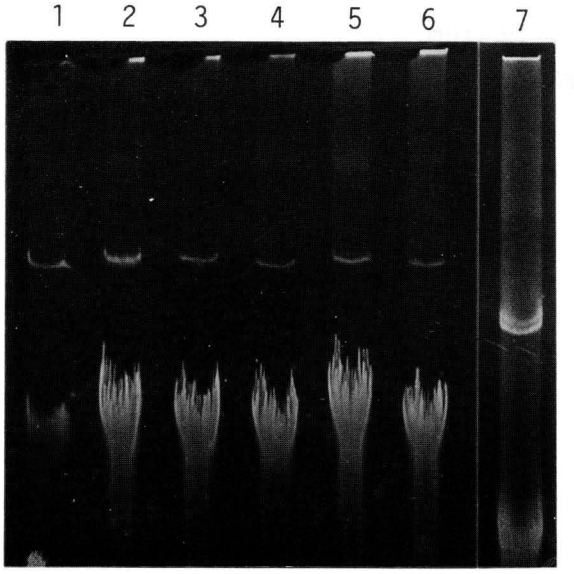


Fig. 1. Agarose Gel Electrophoresis of Crude Plasmids from Pseudomonas C, Moraxella B and their Deletion Mutants, C15 and B86.

1, strain C; 2, strain C plus strain B; 3, strain B; 4, strain C15; 5, strain C15 plus strain B86; 6, strain B86; 7, strain C plus strain C15. The last one was electrophoresed on a separate gel.

Curing of dehalogenating activity

To see if the two dehalogenases of Pseudomonas C are determined by plasmid pUO2, curing experiments were done. Cells were treated with mitomycin C (0.2 μg/ml) or acridine orange (2 μg/ml), and cured cells missing the ability to grow on fluoro- and chloroacetate were sought. However, such cured cells were not obtained at a detectable level of 0.05% in several repeated experiments, while partially cured cells that grew well on fluoroacetate but faintly on chloroacetate were obtained at frequencies of 2 - 4%. The latter cells were found to have lost one dehalogenase, H-2. Mutants deficient in H-2 also appeared spontaneously at almost the same frequency as after curing. The spontaneous loss of H-2 occurred more readily during cultivation in chloroacetate than in peptone medium.

Plasmid DNA wae prepared from ten H-2 deficient mutants, including some spontaneous and some cured ones, and analyzed by gel electrophoresis. They all gave identical bands that migrated somewhat faster than the parental plasmid, pUO2 (Fig. 1, Lane 7, for a representative mutant, C15). In addition, the plasmid was not distinguishable from Moraxella deletion plasmid pUO11 (40.1 Mdal) by migration (Fig. 1, Lanes 4, 5, and 6). These findings suggest that the plasmid of the mutant deficient in H-2, called pUO21, is a deletion derivative of pUO2, and is almost the same size as that of pUO11.

Cleavage analysis with restriction endonucleases

Plasmids pUO2 and pUO21 were purified by CsCl-EtBr equilibrium centrifugation, and digested with the restriction enzymes EcoRI and SalI. The digestion-product patterns obtained by gel electrophoresis are shown in Table I,

TABLE I. Digestion Patterns of pUO2, pUO21, pUO1 and pUO11 by Use of Restriction Enzymes EcoRI and SalI

Alphabetical names of pUO1 fragments were given in the previous section. The sizes (in Mdal) of fragments were estimated against λ-HindIII fragments as standards.

EcoRI				SalI			
pUO2	pUO21	pUO1	pUO11	pUO2	pUO21	pUO1	pUO11
15.0	15.0	A 15.0	15.0	14.8	14.8	A 14.8	14.8
9.6	9.6	B 9.6	9.6	9.6	9.6	B 9.6	9.6
6.6	6.6	C 6.6	6.6	8.2	8.2	C 8.2	8.2
3.1	3.1	D 3.1	3.1			D 2.8	2.8
3.0		E 3.0		2.2	2.2	E 2.2	2.2
2.9	2.9			2.2		F 2.2	
		F 2.4	2.4	2.1	2.1		
1.35	1.35	G 1.35	1.35	1.2		G 1.2	
		H 1.17	1.17	0.95	0.95	H 0.95	0.95
0.50	0.50	I 0.50	0.50	0.76	0.76	I 0.76	0.76

together with the patterns of pUO1 and pUO11. In EcoRI digests of pUO2 and pUO21, 7 fragments are common to both and one fragment (EcoE, 3.0 Mdal) is missing in pUO21. In SalI digests, 7 fragments are common to both and 2 fragments (SalF, 2.2 Mdal, and SalG, 1.2 Mdal) are missing in pUO21. Such homology means that pUO21 is a deletion derivative of pUO2. The fragments missing in pUO21 are the same as the fragments missing in pUO11. This suggests that the same deletion occurred in pUO2 and pUO1.

The cleavage patterns of pUO2 and pUO1 are very similar, as are those of pUO21 and pUO11. However, by careful comparison, pUO1 and pUO2 can be distinguished, as can pUO11 and pUO21. EcoRI-pUO2 digest contains a new 2.9 Mdal fragment, but lacks 2 fragments, EcoF (2.4 Mdal) and EcoH (1.17

MdaI) of pUO1. The other 7 fragments are common to both. SalI-pUO2 digest contains a new 2.1-MdaI fragment instead of fragment SalD (2.8 MdaI) of pUO1. The same differences are observed in pUO11 and pUO21 digests. Therefore, it is evident that pUO2 is a derivative of pUO1, and that pUO2 and pUO21 are about 0.7 MdaI smaller than pUO1 and pUO11, respectively. Comparison of the altered fragments with the original ones suggested that the 0.7-MdaI-deleted region was within SalD of pUO1 and also that it overlapped EcoF and EcoH. A cleavage map of pUO2 was constructed, and is shown in Fig. 2.

Conjugational transfer of pUO2 to Moraxella B123

To demonstrate that pUO2 determines dehalogenases H-1 and H-2 and mercury resistance, and also to compare the expressions of pUO1 and pUO2 in the common host, pUO2 was conjugationally transferred to Moraxella. Recipient strain B123, a plasmid-free derivative of Moraxella sp. B, was resistant to penicillin G, while donor Pseudomonas C was not.

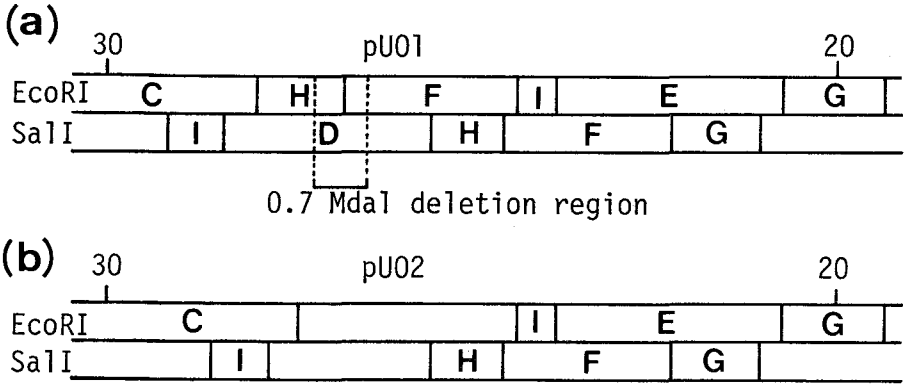


Fig. 2. Parts of the Cleavage Maps of pUO1 (a) and pUO2 (b).
The region deleted in pUO2 is shown by a bar, which overlaps EcoH and EcoF by a length corresponding to 0.7 MdaI, but the precise location is not known.

Therefore, transconjugants were selected on a fluoroacetate plate containing penicillin G (200 μg/ml). The donor and recipient cells separately failed to give colonies on the plate. Transconjugant colonies appeared at a frequency of 3 x 10^{-4} per donor cell. They harbored plasmid DNA with mobility identical to that of pUO2. The plasmid of a representative transconjugant, BC7, was digested with EcoRI and its electrophoretic pattern was compared with those of pUO2 and pUO1 (Fig. 3); the plasmid of BC7 was identified as being pUO2. Transconjugants were found to assimilate fluoro- and chloroacetate and to be resistant to mercury to the same extent as pUO1-carrying Moraxella B (Table II). They were also resistant to penicillin G, kanamycin, streptomycin, rifampicin, and gentamicin, meaning that the host cells were Moraxella B123.

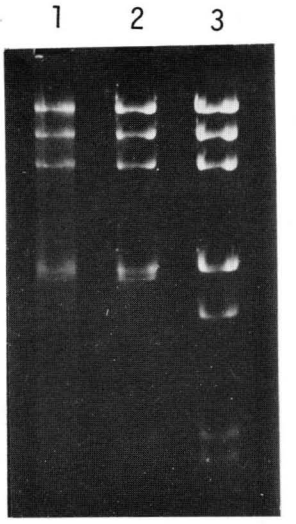


Fig. 3. EcoRI-Digestion Pattern of the Plasmid from Transconjugant BC7.

1, digests of pUO2; 2, digests of BC7 plasmid; 3, digests of pUO1.

TABLE II. Mercury Resistance of Transconjugant BC7 and Other Strains

Each organism was grown for 2 days in L broth supplemented with various concentrations of $HgCl_2$, and a minimal growth-inhibitory concentration was measured.

Strain	MIC of $HgCl_2$ (ug/ml)
Donor, Pseudomonas C (pUO2)	300
Recipient, Moraxella B123	10
Transconjugant, Moraxella BC7 (pUO2)	100
Moraxella B (pUO1)	100

Transconjugants expressed dehalogenases H-1 and H-2, but their regulation of enzyme production was distinctly different from that of donor Pseudomonas C (pUO2) in that transconjugant BC7 produced H-1 inducibly and H-2 constitutively, while Pseudomonas C produced both enzymes constitutively (Table III). In addition, the level of H-2 in the transconjugant was significantly less than that in Pseudomonas C. This productivity of the transconjugant was identical with that of Moraxella B harboring plasmid pUO1, indicating that the regulation of dehalogenase production is dependent on the host cell rather than the plasmid.

As mentioned before, mutants of Pseudomonas C deficient in H-2 appeared spontaneously at a relatively high frequency. However, from the transconjugant such mutants appeared at lower frequencies. This indicates that pUO2 and perhaps pUO1 as well are more easily deleted in Pseudomonas sp. C than Moraxella sp. B, and that the facility of deletion is host-dependent.

TABLE III. Productivity of Dehalogenases in Pseudomonas C, Moraxella B and a Transconjugant, Moraxella BC7

Growth substrate	Specific activity (unit/mg protein) in					
	Pseudomonas C (pUO2)		Moraxella B (pUO1)		Moraxella BC7 (pUO2)	
	H-1	H-2	H-1	H-2	H-1	H-2
Fluoroacetate	0.61	4.8	0.83	0.86	0.69	0.98
Chloroacetate	0.38	3.2	0.09	1.01	0.06	1.04
Peptone	0.63	3.5	0.01	0.46	0.03	0.63

DISCUSSION

A plasmid, pUO2, harbored by Pseudomonas sp. C was shown to be a deletion derivative of pUO1. Moraxella sp. B harboring pUO1 was isolated from industrial wastewater from Saitama Prefecture, while Pseudomonas sp. C was isolated from such wastewater from Osaka Prefecture. This suggests that pUO1 and its derivatives may be distributed over this country and over a range of bacterial genera.

Pseudomonas C (pUO2) produced the same two dehalogenases as H-1 and H-2 of Moraxella B (pUO1). However, Pseudomonas produced both enzymes constitutively, while Moraxella produced H-1 inducibly and H-2 constitutively. In addition, Pseudomonas produced more of H-2 than did Moraxella. At first, I thought that these differences might be due to a 0.7-Mdal deletion in the plasmid pUO2; the regulator gene might be broken by the deletion because the deleted region was adjacent to the H-1 gene (the gene location was estimated later).

However, when pUO2 was transferred from Pseudomonas C to a plasmid-free strain of Moraxella B, pUO2 produced H-1 inducibly and H-2 constitutively in the same amounts as did pUO1. The reverse phenomenon was observed when pUO1 was introduced into another strain of Pseudomonas (Table IV); pUO1 in this organism produced both enzymes constitutively, with H-2 in larger amounts, as did pUO2 in Pseudomonas C. However, pUO1 in Pseudomonas acidovorans was regulated like in Moraxella B. These results mean that the structural difference between pUO1 and pUO2 does not affect regulation of dehalogenase production, and that the regulation is dependent on the host cell.

Plasmid pUO2 in Pseudomonas C was more readily deleted than pUO1 in Moraxella B. However, pUO2 introduced into a Moraxella cell was not so easily deleted as in Pseudomonas C. This indicates that the facility of deletion may also be determined by characteristics of the host cell.

TABLE IV. Expression of pUO1 and pUO2 in Various Hosts

Plasmid	Host	Productivity*	
		H-1	H-2
pUO1	Moraxella B	induc.	constit. (S)
	Pseudomonas acidovorans	induc.	constit. (S)
	Pseudomonas E	constit.	constit. (L)
	Escherichia coli C600	constit.	constit. (L)
pUO2	Pseudomonas C	constit.	constit. (L)
	Moraxella B123	induc.	constit. (S)

* induc., inducible; constit., constitutive. (S) and (L) indicate small and large amounts of H-2 production, respectively.

SUMMARY

Pseudomonas sp. C, a strain capable of assimilating fluoroacetate, was found to harbor a plasmid, pUO2, that determines the production of two haloacetate dehalogenases, H-1 and H-2, as well as mercury resistance. A spontaneous mutant of strain C deficient in H-2 harbored a deletion plasmid, pUO21. pUO2 was very similar to pUO1 and pUO21, pUO11. Cleavage analysis with restriction enzymes revealed that pUO2 was a derivative of pUO1 shortened by 0.7 Mdal, and that pUO21 and pUO11 molecules lacked an identical DNA segment of about 3 Mdal. A cleavage map of pUO2 was constructed and the 0.7-Mdal deleted region was located.

Moraxella B produced H-1 inducibly and H-2 constitutively, whereas Pseudomonas C produced both enzymes constitutively, with a higher ratio of H-2 to H-1. However, pUO2 introduced into Moraxella exhibited the same productivity of the enzymes as pUO1, suggesting that the regulation of dehalogenase production is host-dependent.

CHAPTER III

CLONING AND ANALYSIS OF HALOACETATE DEHALOGENASE GENES

Section 1
Cloning of H-1 and H-2 Genes onto pBR322 and Expression in Escherichia coli

Some studies of plasmids involved in the biodegradation of halogenated compounds have been done, but the genes of dehalogenases have not been analyzed in detail. Recently, Slater et al. (85) isolated an R68.44-prime plasmid carrying a dehalogenase gene from Pseudomonas putida that is capable of degrading chloropropionates. To analyze the genes of haloacetate dehalogenases H-1 and H-2, and to see the genetic relationship among the various dehalogenases, it is necessary to clone the genes of H-1 and H-2.

In this section, I will describe the cloning of the H-1 and H-2 genes onto plasmid pBR322 in E. coli, and refer to the instability of the H-2 clones. The loci of these genes on the pUO1 map and the direction of transcription will also be estimated.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids pUO1 and pUO11, and Moraxella sp. B, were described in Chapter II. E. coli C600 (r_K^-, m_K^-, leu thr trp thi) and plasmid pBR322 were used as the cloning host and vector. Eight plasmids from E. coli V517 were used as size references (pVA517A to H; 35.8, 4.82, 3.67, 3.39, 2.63, 2.03, 1.79, and 1.36 Mdal; 73).

Culture conditions. Strains of E. coli were grown at 37°C in L-broth (1% Polypepton, 0.5% yeast extract, 0.5% NaCl, pH 7.0) or L-agar plates (L-broth containing 1.5% agar). Antibiotic selection was done on L-agar plates containing 50 μg of ampicillin (Ap) or tetracycline (Tc) per milliliter. Moraxella sp. B was grown as described in Chapter II.

Preparation and analysis of plasmid DNA. Plasmid pUO1 was isolated from Moraxella sp. B using the method of Hansen and Olsen (68). pBR322 and its derivatives were extracted from E. coli C600 by the alkaline-SDS method of Birnboim and Doly (86). cc-DNA was purified by CsCl-EtBr equilibrium centrifugation as described in Chapter II.

DNA digestion with various restriction endonucleases was done in the buffers prescribed by the supplier. The restriction fragments were analyzed by agarose gel electrophoresis as described in Chapter II.

Ligation and transformation. pUO1 and pBR322 were separately digested to completion with the appropriate restriction enzyme and then kept at 65°C for 10 min. The two digests were mixed, precipitated with ethanol, and dissolved in the ligation buffer (66 mM Tris-HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 0.5 mM ATP). Ligation was carried out using T4 DNA ligase at 15°C overnight. Transformation of $CaCl_2$-treated E. coli was done using the method of Cohen et al. (87). Transformants with hybrid pBR322 carrying a foreign DNA inserted at the restriction site of SalI, HindIII or BamHI were selected by ampicillin resistance (Ap^r) and tetracycline susceptibility (Tc^s), and then examined for H-1 and H-2 activities by a convenient method described below.

Transformants with hybrids carrying EcoRI inserts were selected first on ampicillin plates and then examined for dehalogenase activity. On occasion, H-2^+ transformants were selected by forming yellow colonies on L-agar plates containing 0.2% monochloroacetate and bromothymol blue (50 μg/ml), because the dehalogenation of monochloroacetate lowers the pH of the medium.

Assay of H-1 and H-2 activities. Dehalogenase activity was assayed by measuring glycolate produced from monofluoro- or monochloroacetate using the colorimetric method of Dagley and Rodgers. Cells grown overnight in 5 ml of L-broth or on an L-agar plate were harvested, washed, and suspended in 2 ml of 0.05 M phosphate buffer, pH 7.0, containing 5 mM 2-mercaptoethanol. One drop of toluene was added and the suspension was shaked at 30°C for 30 min. A 50-μl aliquot of the toluenized cell suspension was added to an assay mixture containing 100 μl of 0.1 M Tris-HCl, pH 9.0, and 50 μl of 0.03 M monofluoro- or monochloroacetate. After a 20-min incubation at 30°C, the reaction was stopped by the addition of 0.5 ml of 2% chromotropic acid and 5 ml of conc. H_2SO_4, and then the mixture was heated in boiling water for 30 min. The blue color that developed was measured in terms of the optical density at 570 nm.

Chemicals and reagents. The restriction endonucleases SalI, EcoRI, HindIII, SmaI, and BamHI, and the T4 DNA ligase were purchased from the Takara Shuzo Co., Ltd., Kyoto, Japan. Ampicillin was obtained from the Meiji Seika Kaisha, Ltd., Tokyo, Japan, and tetracycline from the Sigma Chemical Co., St. Louis, U.S.A. Other chemicals were commercial products.

RESULTS AND DISCUSSION

Expression of pUO1 in E. coli

The $CaCl_2$-treated cells of E. coli C600 were transformed with pUO1 DNA prepared from Moraxella sp. B. Transformants selected on L-agar plates containing $HgCl_2$ (30 μg/ml) had the activities of dehalogenases H-1 and H-2. However, they could not grow on monofluoro- or monochloroacetate as the source of carbon and energy, though they could grow on glycolate. This is probably due to the toxic action of haloacetate, such as the "lethal synthesis" of fluorocitrate, an inhibitor of the Krebs cycle.

Plasmid DNA was isolated from the transformant cells grown freshly on peptone, and its electrophoresis showed that it included DNA of both pUO1 and its deletion mutant pUO11 in almost the same amounts. To examine the stability of pUO1 in E. coli, pUO1-harboring cells were cultured in L-broth for a long period (40 hr), and then spread on L-agar; plasmid in each colony was analyzed. About half of the colonies tested contained pUO1, pUO11, or both, and the remaining colonies had no plasmids. The pUO11-harboring cells were about half the population of plasmid-inheritors. This indicates that pUO1 is very unstable in E. coli; it readily disappears or is deleted. A supplement of monochloroacetate in the growth medium allowed an increase in the population of pUO1-carrying cells.

Cloning of H-1 and H-2 genes of pUO1 onto pBR322

After the pUO1 DNA was digested with restriction endonucleases SalI, EcoRI, HindIII, and BamHI, the resulting fragments were ligated to pBR322 DNA, and provided for transformation of E. coli C600.

Cloning of SalI-pUO1 digests. Four Ap^r Tc^s transformants were obtained. Two of them produced the H-1 enzyme, and one produced the H-2 enzyme. Analysis of their plasmids (Fig. 1a) showed that the two $H\text{-}1^+$ clones (Lanes 2 and 3) had an identical 5.4-Mdal plasmid, which was cleaved by SalI into two fragments of 2.6 and 2.8 Mdal, corresponding to pBR322 DNA and the SalI-D fragment of pUO1. The names and loci of the restriction fragments of pUO1 are given in Fig. 2. The $H\text{-}2^+$ clone (Lane 4) carried a 3.8-Mdal hybrid plasmid that contained a 1.2-Mdal insert corresponding to the SalI-G fragment. Another clone (Lane 5) had a 4.8-Mdal hybrid plasmid containing a 2.2-Mdal insert corresponding to the SalI-E fragment. The inserted fragments were identified by further restriction analysis. The H-1 hybrid plasmid containing SalD was tentatively called pBRSD1 and the H-2 hybrid containing SalG, pBRSG2.

Cloning of EcoRI-pUO1 digests. Two $H\text{-}1^+$ clones and one $H\text{-}2^+$ clone were obtained. The $H\text{-}1^+$ clones had identical hybrid plasmids containing an insert of the EcoRI-F fragment (2.4 Mdal) (Fig. 1b). The $H\text{-}2^+$ clone had a hybrid plasmid with the EcoRI-E fragment (3.0 Mdal). The H-1 and H-2 hybrid plasmids were tentatively named pBREF1 and pBREE2.

Cloning of HindIII-pUO1 digests. HindIII-D (1.48 Mdal) and -E (0.60 Mdal) fragments were cloned, but these clones did not have the dehalogenase activity. Then, the HindIII-C fragment (3.0 Mdal), which was thought to carry the H-2 gene, was purified by agarose gel electrophoresis and cloned onto pBR322 (Fig. 1c, Lane 4). This clone expressed the H-2 enzyme, and the hybrid plasmid was tentatively named pBRHC2.

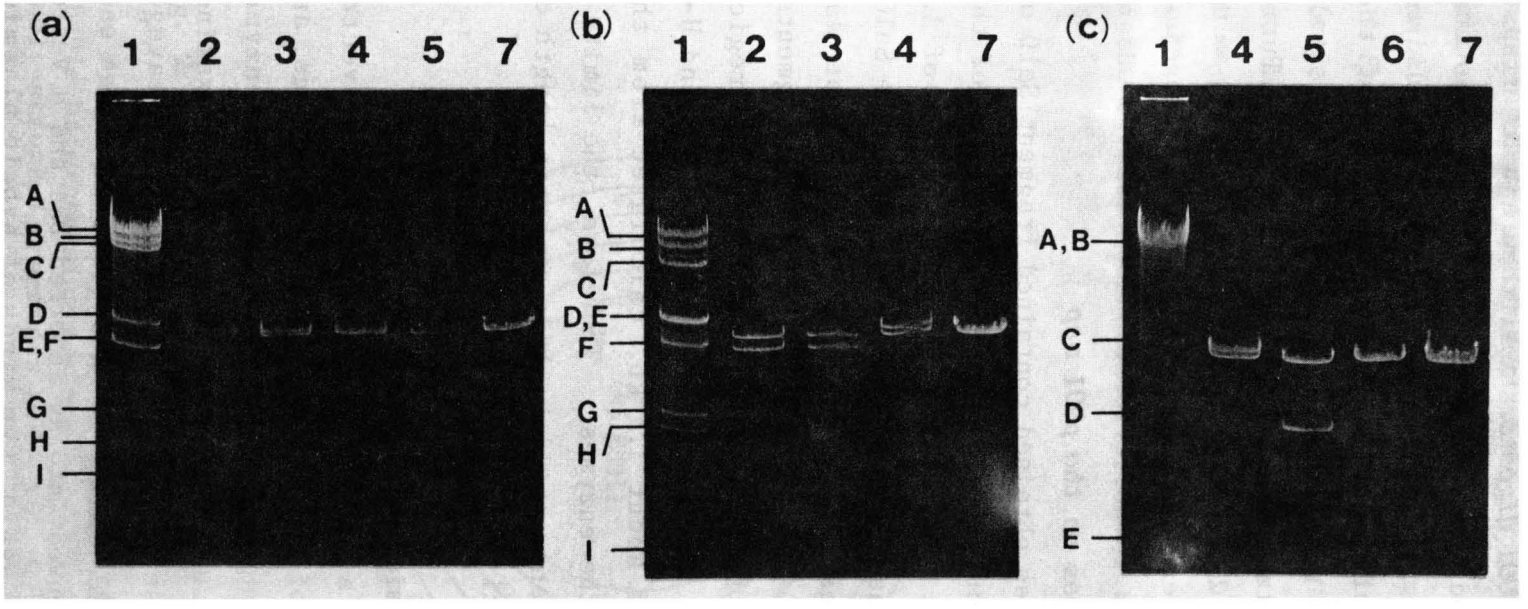


Fig. 1. Restriction Analysis of Hybrid pBR322 Containing (a) SalI-Digests, (b) EcoRI-Digests, and (c) HindIII-Digests of pUO1 DNA.

pUO1, pBR322 and hybrid plasmids were digested by (a) SalI, (b) EcoRI, and (c) HindIII, and analyzed by gel electrophoresis. 1, pUO1; 2 and 3, H-1$^+$ hybrids; 4, H-2$^+$ hybrids; 5 and 6, other hybrids; 7, pBR322.

Cloning of BamHI-pUO1 digests. Fifteen Ap^r Tc^s transformants were obtained, but they all did not express the dehalogenase. Plasmids of these transformants were found to be deletion mutants of pBR322, lacking segments of the various lengths around the insertion site (the BamHI site); the range of plasmid sizes was from 1.4 to 2.5 Mdal. Purified fragments BamC (2.2 Mdal) and BamD (1.22 Mdal) were cloned into pBR322, but they did not express the dehalogenase.

Loci of H-1 and H-2 genes on the pUO1 map

Since the H-1 clones obtained contained fragment SalD or EcoF, the H-1 gene should be located in the overlap of the two fragments (Fig. 2); the overlap region has a span of 1.6 kb. The H-2 enzyme was expressed by the clones of the SalG, EcoE, and HinC fragments. Therefore, the H-2 gene was within SalG (1.8 kb), a region common to the three fragments, and as expected, the locus was within the 3 Mdal-region deleted in pUO11. The structural genes of H-1 and H-2 both have a length of about 1.3 kb, calculated from the molecular weights of the enzymes. Therefore, the limits I have set for the two genes still include an extra length of 0.3 and 0.5 kb, respectively.

Orientation and expression of the inserted genes

The insertion of a restriction fragment into a vector DNA occurs in either of the two possible orientations, and can be judged by cleavage with another restriction enzyme; in practice the orientations of EcoF and EcoE were examined by SalI cleavage, those of SalD and HinC by EcoRI cleavage, and that of SalG by SmaI-BamHI double cleavages. When each hybrid plasmid was cleaved into an inserted DNA and a vector DNA, and then ligated again, two kinds of hybrid plasmids

with the fragment inserted in opposite orientations were obtained. The EcoF-inserted hybrid expressed the H-1 gene regardless of the orientation of the insert, while the hybrid with SalD inserted in the opposite orientation to the parent hybrid, pBRSD1, did not express the H-1 gene. This suggests that the H-1 promoter is contained in the EcoF fragment but not in the SalD fragment, so that in the map drawn in Fig. 2, the promoter is located at the right of the H-1 gene limit, and the direction of transcription of the H-1 gene is clockwise.

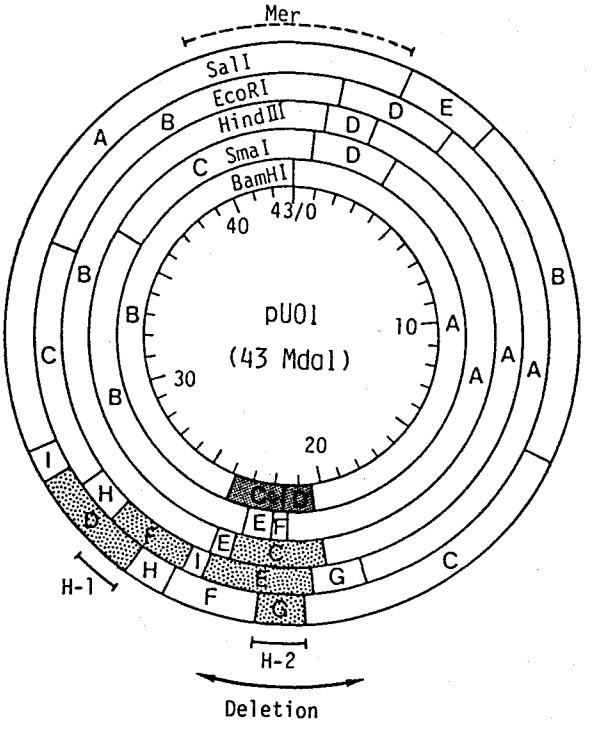


Fig. 2. Locations of the H-1 and H-2 Genes on the Restriction Map of pUO1.

Hybrids containing coarsely-stippled fragments are capable of expressing either H-1 or H-2, and ones containing finely-stippled fragments were not.

Why did the parent SalD hybrid express the H-1 gene without its own promoter? This hybrid carried the H-1 structural gene downstream from the promoter of the Tc^r gene (Fig. 3). When the fragment E2 that contained the H-1 structural gene and the Tc promoter was cleaved out with EcoRI and newly inserted into an EcoRI-cleaved pBR322 DNA, two kinds of hybrid plasmids with the fragment in opposite orientations expressed the H-1 gene equally. On the other hand, when the fragment ES2, which contained the H-1 structure gene alone, was subcloned into an EcoRI-SalI doubly cleaved pBR322 DNA that lacked the Tc promoter, the resulting hybrid plasmids did not express the H-1 gene. Therefore, the H-1 expression of the SalD or E2 hybrid can be explained as being caused by the readthrough transcription initiated at the Tc promoter of pBR322. Quantitative expression of the H-1 gene of these hybrids was about three times that of

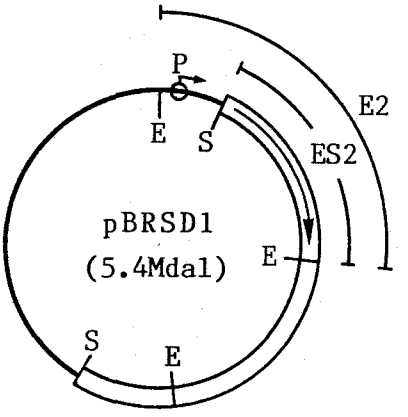


Fig. 3. Subcloning of the H-1 Structural Gene from Hybrid pBRSD1 Containing the SalD Fragment.

The thick line indicates pBR322 DNA and the open box indicates the inserted SalD fragment. The promoter of the Tc gene is indicated by P, and the direction of transcription by arrows. Restriction sites of SalI and EcoRI are expressed by S and E, respectively. ES2 and E2 are the fragments subcloned.

pBREF1, which suggests that the Tc promoter is more efficient in E. coli than the H-1 one.

The SalG hybrid plasmid expressed the H-2 gene equally, without regard to the orientation of the insertion. Therefore, the promoter of the H-2 gene may reside in the SalG fragment.

Stability of H-1 and H-2 hybrid plasmids

After the H-1 and H-2 clones were cultured serially twice in L-broth, the preservation of the plasmid in the cell was examined using the criterion of the Ap resistance of the cell. The results (Table I) indicate that the H-1 hybrid plasmids, pBRSD1 and pBREF1, were maintained stably in E. coli, while the H-2 hybrids, pBRSG2, pBREE2, and pBRHC2, were very unstable. It was confirmed that the Ap^r cells maintained the plasmids and expressed the dehalogenase, and that the Ap^s cells did not have the plasmid. As an exceptional

TABLE I. Stability of Hybrid Plasmids

After each clone was grown in L-broth for 2 days, the populations of Ap^r and Ap^s cells were determined by the replica method. It was confirmed that Ap^s cells carried no plasmid.

Hybrid plasmid	Fragment inserted (MdaI)	Phenotype	Retention of plasmid (%)
pBRSD1	SalD (2.8)	H-1 Ap^r	99
pBREF1	EcoF (2.4)	H-1 Ap^r Tc^r	97
pBRSG2	SalG (1.2)	H-2 Ap^r	5
pBREE2	EcoE (3.0)	H-2 Ap^r Tc^r	1
pBRHC2	HinC (3.0)	H-2 Ap^r	4
pBR322	-	Ap^r Tc^r	100

case, Ap^r $H\text{-}2^-$ mutants arising from the clones of pBREE2 and pBRHC2 contained the deletion plasmids, which were lacking not only the inserted DNA but also various lengths of the pBR322 DNA around the insertion site. Similar deletions were observed when cloning the BamC fragment.

pBREE2, a hybrid containing the EcoE fragment, was extremely unstable. However, when the small segment (0.6 Mdal) indicated in Fig. 4 was deleted enzymatically from pBREE2, the plasmid became stable. The region deleted from the inserted EcoE, nearly equivalent to fragment HinE, was coincident with an end of the specific 3-Mdal area that is readily deleted from pUO1. The BamC fragment that includes this region made its hybrid plasmid unstable. Another end of the 3-Mdal deletion region was included in the HinC fragment, whose clone pBRHC2 was unstable too. These findings

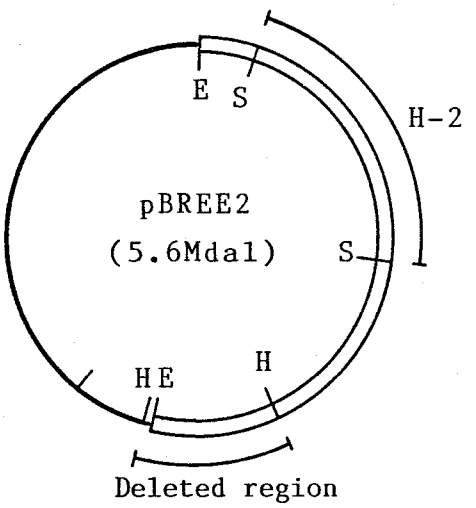


Fig. 4. A Map of Unstable Hybrid pBREE2 Containing the EcoE Fragment.

The thick line and open box indicate pBR322 DNA and the inserted EcoE fragment, respectively. Restriction sites of SalI, EcoRI, and HindIII are expressed by S, E, and H. The locus of the 0.6 Mdal segment that was cut off by HindIII is indicated as a deleted region.

suggested that the instability of the hybrid plasmids containing EcoE, BamC, and HinC might be caused by a particular DNA structure participating in the deletion of the 3-MdaI segment from pUO1.

SUMMARY

The H-1 and H-2 genes of plasmid pUO1 were cloned onto plasmid pBR322 in E. coli. Analysis of the cloned DNA fragments revealed the loci of both genes on the pUO1 map and also the direction of transcription. Two different hybrid pBR322 containing the H-1 gene were maintained stably in E. coli, while three hybrids containing the H-2 gene were so unstable that they readily disappeared or changed into various deletion mutants. The instability of the H-2 hybrid plasmids might be caused by a certain DNA structure adjacent to the H-2 gene that may be concerned in the deletion of the H-2 gene from pUO1.

Section 2
Heterology Between the H-1 and H-2 Genes

It is known that enzyme evolution is initiated by the tandem duplication of a gene, followed by multiple mutation of either gene copy, resulting in the creation of a modified enzyme that acts on novel substrates (35).

Dehalogenases H-1 and H-2 differ slightly in their substrate specificity, but are very similar in molecular weight and isoelectric point. In addition, the H-1 and H-2 genes are in tandem to each other in the pUO1 DNA. These findings suggest that there is an evolutional relationship between the two genes.

In this section, the homology of the H-1 and H-2 genes is examined using the Southern hybridization method (88).

MATERIALS AND METHODS

Purification of DNA fragments. DNA cleaved by a restriction enzyme was electrophoresed on an agarose gel (Sigma, Agarose Type V), and stained with EtBr. The gel strips containing the appropriate fragment bands were cut out and DNA was recovered from them by electroelution (82). The DNA eluted was purified by phenol extraction and ethanol precipitation, and dissolved in either TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) or distilled water.

α-^{32}P-labeling of DNA by nick translation. The Nick Translation System of New England Nuclear (NEK-004C), including [α-^{32}P]-dCTP, was used to label a DNA probe. Nick translation was done following the instruction manual of the supplier. The composition of the reaction mixture was as

follows: 5 µl of [α-^{32}P]-dCTP (800 Ci/mmol, 10 µCi/µl), 5 µl of nick translation buffer (Tris-HCl, $MgCl_2$, bovine serum albumin, and dithiothreitol, pH 7.4), 4 µl of cold deoxynucleoside triphosphate mixture (dATP, dGTP, and dTTP, each at 0.1 mM), 2 µl of DNA (about 0.5 µg), 5 µl of water, 2 µl of DNA polymerase (0.6 units/µl), and 2 µl of DNase I (0.2 units/ml). It was incubated at 14°C. Incorporation of ^{32}P-dCTP into DNA was followed by counting the radioactivity of acid-insoluble ^{32}P at appropriate times. Maximum incorporation was achieved within 2-3 hr. To separate labeled DNA from the free ^{32}P-dCTP, the reaction mixture was put on a Bio-Gel P30 column (1 x 20 cm) equilibrated with TE buffer and eluted with the same buffer. About 0.5 ml of effluent samples were collected in microfuge tubes and radioactive DNA fractions were detected with a hand monitor.

Transfer of DNA to nitrocellulose filter. Blotting was done using the procedure described in the Manual for Genetic Engineering (89). DNA cleaved with a restriction endonuclease was electrophoresed on a 1% agarose gel, stained with EtBr, and photographed. The gel was immersed in 0.25 N HCl at room temperature for 30 min to break any large DNA, and rinsed with water. Then, alkaline denaturation of DNA was done by immersing the gel in 0.5 N NaOH containing 1.5 M NaCl for 30 min. The gel was neutralized with 0.5 M Tris-HCl (pH 7.5) containing 1.5 M NaCl for 1 hr, and then set in an electrophoretic blotting apparatus (Toyo Kagaku Sangyo Co., Model ETB-15) together with a nitrocellulose filter sheet. Blotting was carried out in Tris-acetate buffer (40 mM Tris, 29 mM sodium acetate, and 5 mM EDTA, pH 8.2, adjusted with acetic acid) at 25 V for 3-4 hr in an ice bath. The filter was rinsed in 2 x SSPE (20 mM $Na_{1.5}H_{1.5}PO_4$, 0.36 M

NaCl, and 1 mM Na_2EDTA, pH 7.0), and dried in a vacuum dessicator at 80°C for 2 hr.

DNA-DNA hybridization. Hybridization was done as described in the Manual for Genetic Engineering (89). ^{32}P-labeled DNA probes were denatured by incubation at 95°C for 10 min. DNA-bound filters were placed in a heat-sealable plastic bag and treated by incubation at 42°C for 1 hr in prehybridization solution containing 50% (v/v) formamide, 5 x SSPE, 5 x BFP (0.1% bovine serum albumin, 0.1% Ficoll of M.W. 400,000, and 0.1% polyvinyl pyrrolidone), 1% glycine, and denatured, sonicated carrier DNA (100 µg/ml). Upon removal of the prehybridization solution, the denatured DNA probe and hybridization solution containing 50% formamide, 5 x SSPE, 1 x BFP, denatured, sonicated carrier DNA (100 µg/ml), and 0.3% SDS were added to the bag with the filter. Hybridization was done at 42°C for 24 hr. The filter was washed four times in a solution containing 20 mM $Na_{1.5}H_{1.5}PO_4$, 0.2% SDS, and 1 mM Na_2EDTA at 37°C, and then dried.

Autoradiography. The filter was placed on X-ray film (Fuji Film, Medical RX) in a film cassette equipped with a intensifying screen (Fuji Film, Hi-Screen H3) in a -70°C freezer for 12-60 hr.

RESULTS

Hybridization using the H-1 gene as a probe

It was described in the preceding section that the H-1 structural gene is located in a overlapping segment (1.6 kb) between fragments SalD and EcoF of the pUO1 map. The segment was purified, labeled with ^{32}P, and used as an H-1 gene

probe. Purified pUO1 DNA was digested with SalI, EcoRI, or both, and electrophoresed on a agarose gel. After blotting on a nitrocellulose filter, Southern hybridization with the H-1 gene probe was conducted. The results (Fig. 1A) indicated that the probe hybridized with fragments SalD and EcoF and with an overlap of them, but not with SalG and EcoE, which contain the H-2 gene. The positions of these fragments in the pUO1 map are illustrated in Fig. 1B.

Hybridization using the H-2 gene as a probe

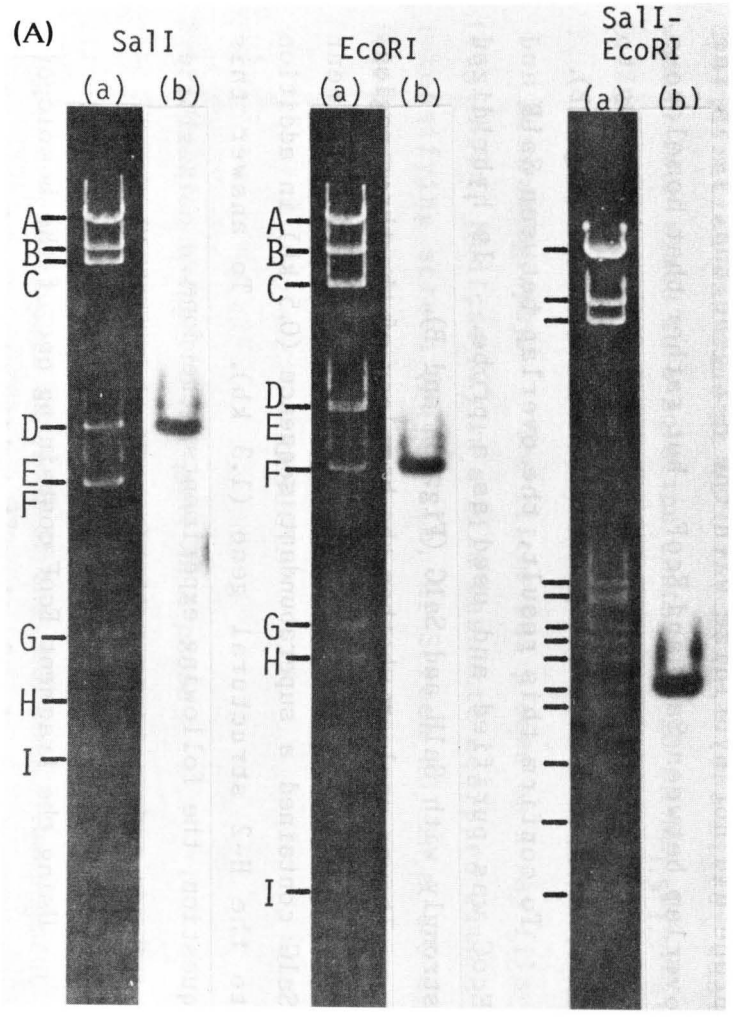
Fragment SalG (1.8 kb) was labeled and used as an H-2 gene probe in Southern hybridization with SalI- or EcoRI-digested pUO1. The probe hybridized with fragments SalH, SalD, EcoF, and EcoC in addition to SalG and EcoE (Fig. 2A). By taking into consideration the results of hybridization with SalI-EcoRI double digests, it was seen that the H-2 gene probe did not hybridize with the H-1 gene that is in the overlap between SalD and EcoF, but rather that homologous sequences were present in three regions corresponding to SalG, SalH, and an overlap between SalD and EcoC (Fig. 2B).

To confirm this result, the overlap between SalD and EcoC was purified and used as a probe. It hybridized strongly with SalH and SalG (Fig. 3A and B).

It was unclear whether the homology of the three regions was owing to the H-2 structural gene, because the fragment SalG contained a superabundant sequence (0.5 kb) in addition to the H-2 structural gene (1.3 kb). To answer this question, the following experiments were done.

Location of the homologous sequence in fragment SalG

Using the fragment EcoF containing one of the homologous sequences as a probe, Southern hybridization was done against



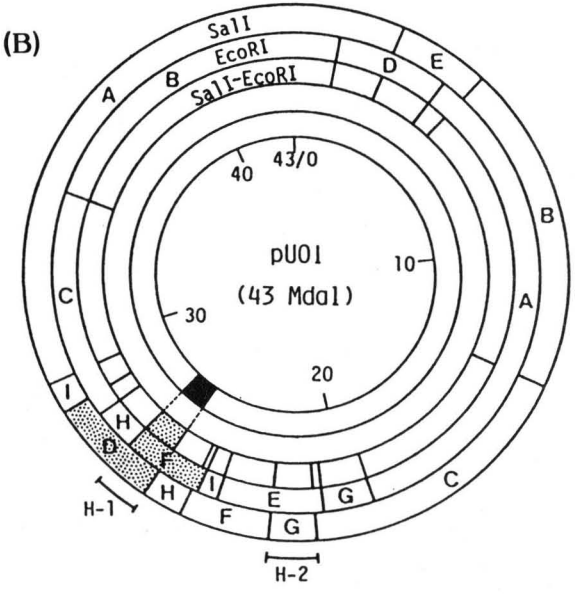


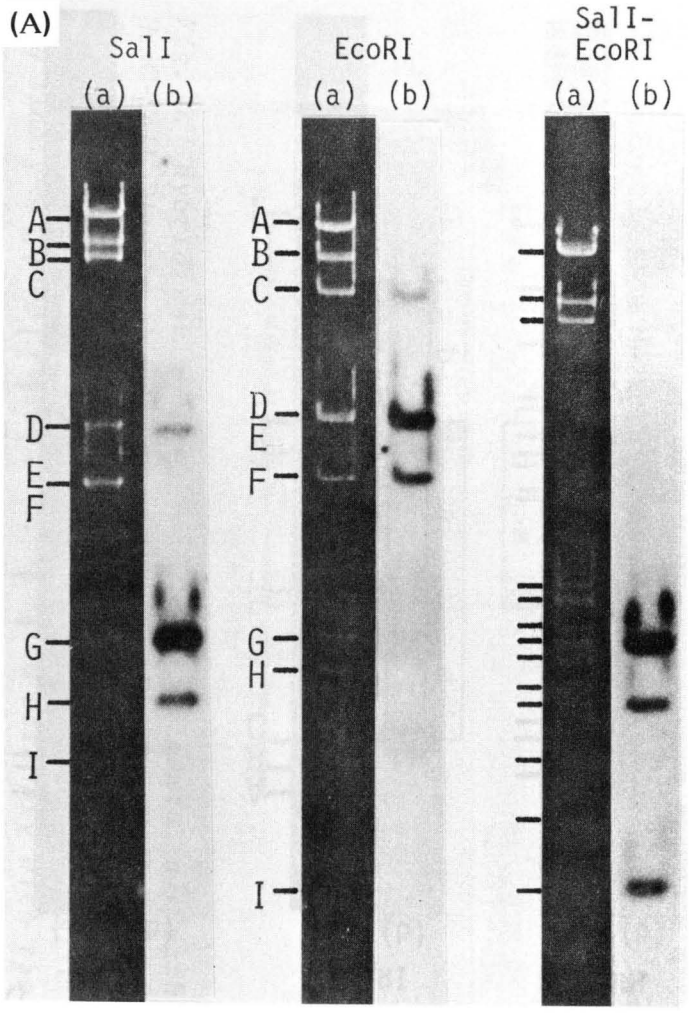
Fig. 1. (A) Southern Hybridization Using the H-1 Gene as a Probe.

(a), electrophoretic patterns of restriction fragments of pUO1

(b), autoradiograms of hybrids on nitrocellulose filter

(B) Loci of Fragments that Hybridized with the H-1 Gene Probe.

The fragment used as a probe is blacked out, and the hybridized fragments are stippled.



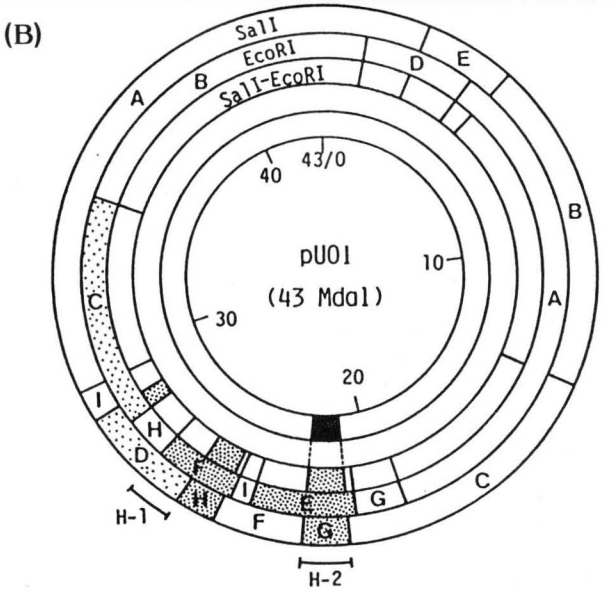


Fig. 2. (A) Southern Hybridization Using the H-2 Gene (Sa1G) as a Probe.

(a), electrophoretic patterns of restriction fragments of pUO1

(b), autoradiograms of hybrids on nitrocellulose filter

(B) Loci of Fragments that Hybridized with the Sa1G Probe.

The blacked-out fragment was the probe, the finely-stippled ones hybridized strongly, and the coarsely-stippled ones, weakly.

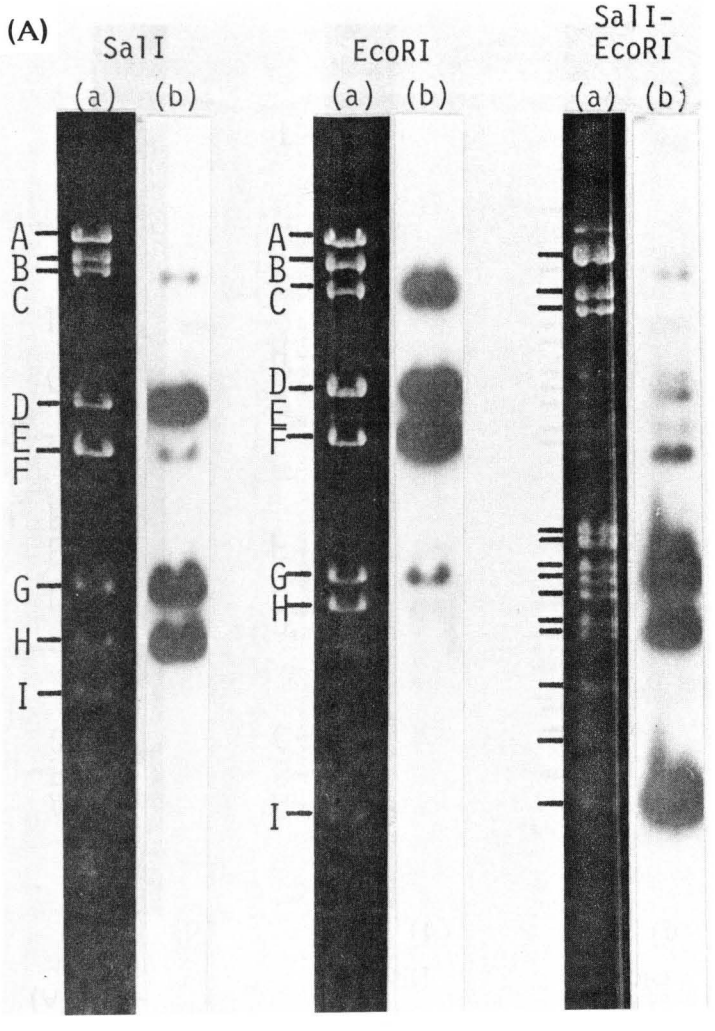


Fig. 3. (A) Southern Hybridization Using the Overlap Between SalD and EcoC as a Probe.

(a), electrophoretic patterns of restriction fragments of pUO1

(b), autoradiograms of hybrids on nitrocellulose filter

(B) Loci of Fragments that Hybridized with the Overlap of SalD-EcoC.

Stippled fragments hybridized with the probe.

HincII digestion products of SalG, which consisted of three fragments, a (0.8 kb), b (0.7 kb), and c (0.3 kb). The probe hybridized strongly with the fragment c and weakly with the fragment a, but not at all with the fragment b (Fig. 4). This indicate that a large part of the homologous sequence resides in the fragment c. When the fragment c was used as a probe, it hybridized with SalG, SalH, and SalD of pUO1-SalI digests; the fragment b used as a probe did not hybridize with any fragments other than SalG (data not shown).

Against SalI or EcoRI digests of fragment HinC, which includes the area adjacent to SalG (Fig. 4), the EcoF probe that contains the homologous sequence hybridized faintly with fragment c of SalI digests but not with fragment b of EcoRI digests, suggesting that the homologous sequence extends slightly over the SalG to the right as seen in Fig. 4. Therefore, it seems that the H-2 gene is not involved in the homology of the three regions. The homologous sequence may be adjacent to the H-2 gene.

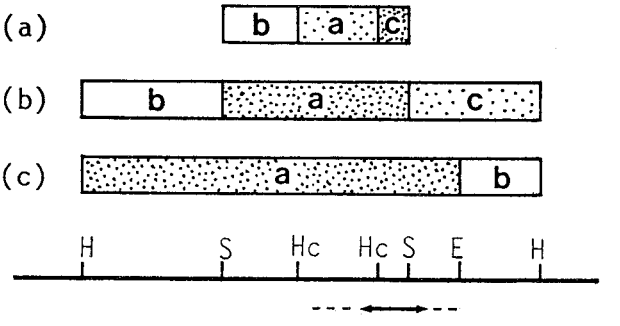


Fig. 4. Hybridization with SalG-HincII Digests (a), with HinC-SalI Digests (b), and with HinC-EcoRI Digests (c) Using EcoF as a Probe.

Hc, a HincII site; S, a SalI site; E, an EcoRI site. The finely-stippled fragments hybridized strongly with the probe, and the coarsely-stippled fragments, weakly. The arrow indicates the limit of the homologous sequence.

DISCUSSION

The H-1 and H-2 genes did not hybridize with each other either at 65°C in an aqueous solution or at 42°C in 50% formamide. Therefore, I concluded that the base sequences of the two genes are not homologous and so that there is no evolutional relationship between the H-1 and H-2 enzymes. Possibly the H-2 gene has been transposed from another replicon to pUO11. This possibility will be discussed in the next section.

Unexpected homologous sequences were found in three regions of the DNA of pUO1. The regions were near the loci of the H-1 and H-2 genes; two were adjacent to either the H-1 or the H-2 gene.

Each sequence had a length of about 0.5 kb and was repeated after intervals of about 5 kb with either the H-1 or the H-2 gene in between (Fig. 5). The functions of these sequences are not known.

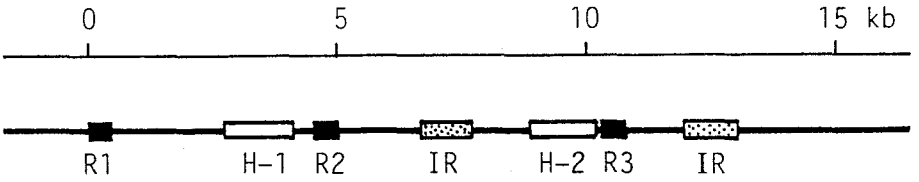


Fig. 5. Loci of the Repeated Sequences on pUO1.
R1, R2, and R3 are the repeated sequences, and IR is an inverted repeat, which will be described in the following section.

SUMMARY

Southern hybridization experiments demonstrated heterology of the base sequences of the H-1 and H-2 genes, suggest-

ing that there is no evolutional relationship between the H-1 and H-2 enzymes. There were homologous sequences in three regions of pUO1 DNA. Each sequence had a length of about 0.5 kb and was repeated after intervals of about 5 kb with either the H-1 or the H-2 gene in between.

Section 3
Transposon-like Structure of the H-2 Gene

Plasmid pUO1 readily converted to the deletion plasmid pUO11 by losing a specific segment of about 3 Mdal, which contains the H-2 gene. This deletion occurred independently of the rec function of the host. I mentioned before that the hybrid pBR322 with pUO1 segments near the H-2 gene were very unstable in E. coli (rec^-). I thought that the instability of the hybrids might be caused by a specific DNA structure derived from the pUO1 segment. The structure also appeared to be implicated in the deletion of the 3-Mdal segment of pUO1. It is well-known that insertion sequences (IS) and transposons bring about their own excision from the plasmid DNA and also delete nearby segments of DNA (90). Transposons are also known to be bracketed by inverted repeat sequences. To see if the H-2 gene is on a transposon, the DNA structure near the H-2 gene was analyzed by Southern hybridization and intra-strand annealing.

MATERIALS AND METHODS

Intra-strand annealing. Annealing of DNA and electron microscopic observation of annealed DNA were done by the method described by Davis et al. (89). The DNA fragments were converted to single-stranded DNA by alkaline or heat denaturation, and after neutralization, the DNA (0.1-0.25 µg/50 µl) was incubated in 50% formamide at 25°C for 30 min. The DNA preparation (10 µl) was mixed with 10 µl of 1 M Tris-HCl (pH 8.5) containing cytochrome c (0.5 mg/ml) and 0.1 M Na_2EDTA, 45 µl of H_2O, and 35 µl of formamide, and spread over the hypophase containing 10 mM Tris-HCl (pH 8.5), 1 mM

Na_2EDTA, and 10% formamide. Mounting on a polyvinyl-formvar-coated grid, staining with uranyl acetate, and shadowing with platinum-palladium were done as described in Chapter II. The DNA was photographed under a Hitachi H-600 electron microscope. The length of single-stranded DNA was determined using denatured ColEI DNA as the internal standard (6.4 kb).

Nick translation and Southern hybridization. The procedures used here were the same as those described in Section 2.

RESULTS AND DISCUSSION

Hybridization analysis of repeated sequences

Repeated sequences near the H-2 gene were searched for by Southern hybridization. The overlap (1.5 Mdal) of the fragments SalF and EcoE, in which one endpoint of the 3-Mdal deletion is located, was cut out from pUO1 DNA, purified, labeled with ^{32}P by nick translation, and used as a probe in the hybridization analysis of SalI and EcoRI digests of pUO1 DNA. The probe hybridized with SalC and EcoG in addition to SalF and EcoE (Fig. 1A), indicating that repeated sequences are present with the H-2 gene between (Fig. 1B).

To limit further the locus of the sequence included in EcoG, analysis using the same probe was done of the HindIII-digestion products of EcoG. As shown in Fig. 2, the right fragment (0.8 Mdal) hybridized more strongly than another fragment (0.5 Mdal) did. Therefore, the segment is located toward the right part of EcoG.

To locate another sequence, EcoG (1.35 Mdal) was used as a probe and hybridization was done against the HindIII-digests of the overlap of EcoE and SalF. As seen in Fig. 2,

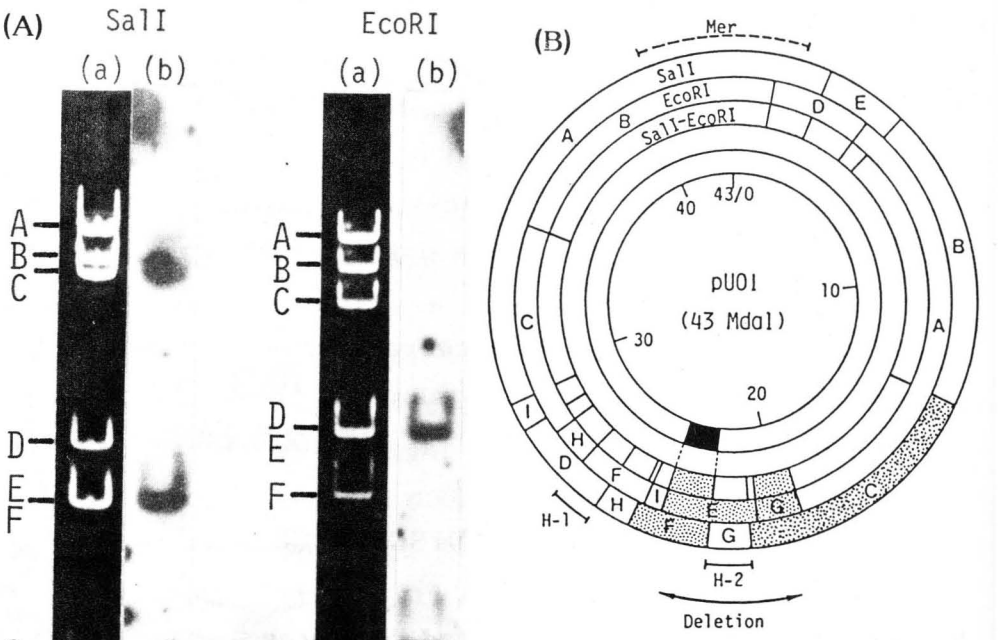


Fig. 1. (A) Southern Hybridization Against SalI and EcoRI Digests of pUO1 Using the Overlap of SalF and EcoE as a Probe.

(a) Electrophoretic patterns of restriction fragments of pUO1

(b) Autoradiograms of hybrids on nitrocellulose filter

(B) Loci of Fragments that Hybridized with the Overlap of SalF-EcoE.

Stippled fragments hybridized with the probe (the blacked out fragment).

the left fragment (0.6 Mdal) hybridized strongly and the right fragment (0.9 Mdal), weakly. These findings suggest that the repeated sequences were located in regions corresponding to the end-points of the 3-Mdal deletion.

Inverted repeat sequences putting the H-2 gene between

Inverted repeat sequences on plasmid DNA undergo intra-

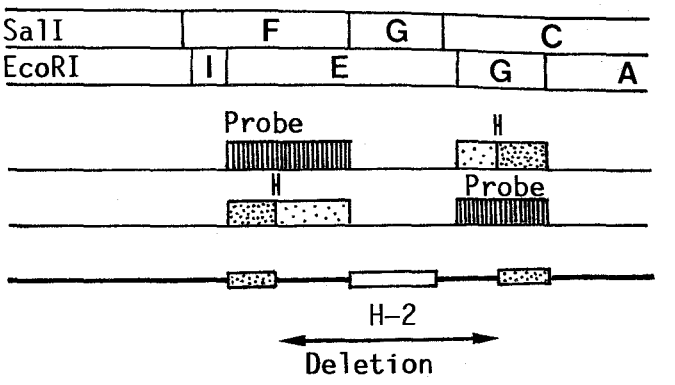


Fig. 2. Limitation of Repeated Sequences Using Hybridization Analysis.

The finely-stippled fragment was hybridized strongly with the probe (striped fragment), and the coarsely-stippled one, weakly.

strand annealing in formamide, with the consequent formation of hairpin loop structures (90). pUO1 DNA was cleaved by restriction enzyme XhoI into four fragments (16.4, 13.6, 9.6, and 1.9 Mdal), whose loci on the pUO1 map were estimated (Fig. 3). The 13.6-Mdal fragment containing the H-2 gene was suitable for an annealing experiment, because intact pUO1 DNA is too large to maintain stably the small loop formed.

The DNA fragments were denatured into single-stranded DNA and allowed to anneal in 50% formamide. Electron microscopic observation showed there was formation of loop structures with short duplex stems (Fig. 4). The sizes of loops and stems were measured using single- and double-stranded ColEI DNA as the internal standard (6.4 kb). (It was difficult to measure precisely the length of single-stranded DNA because it was quite crinkled.) The loop was about 5.4 kb in length on the average, and the stem was about 1 kb long. The stem is formed by complementary base pairing. Therefore,

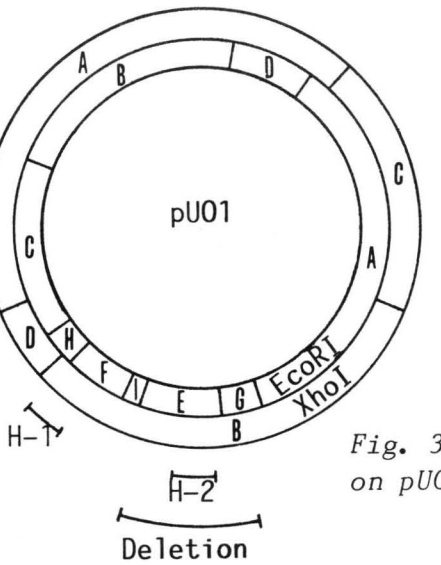


Fig. 3. Cleavage Sites of XhoI on pUO1.

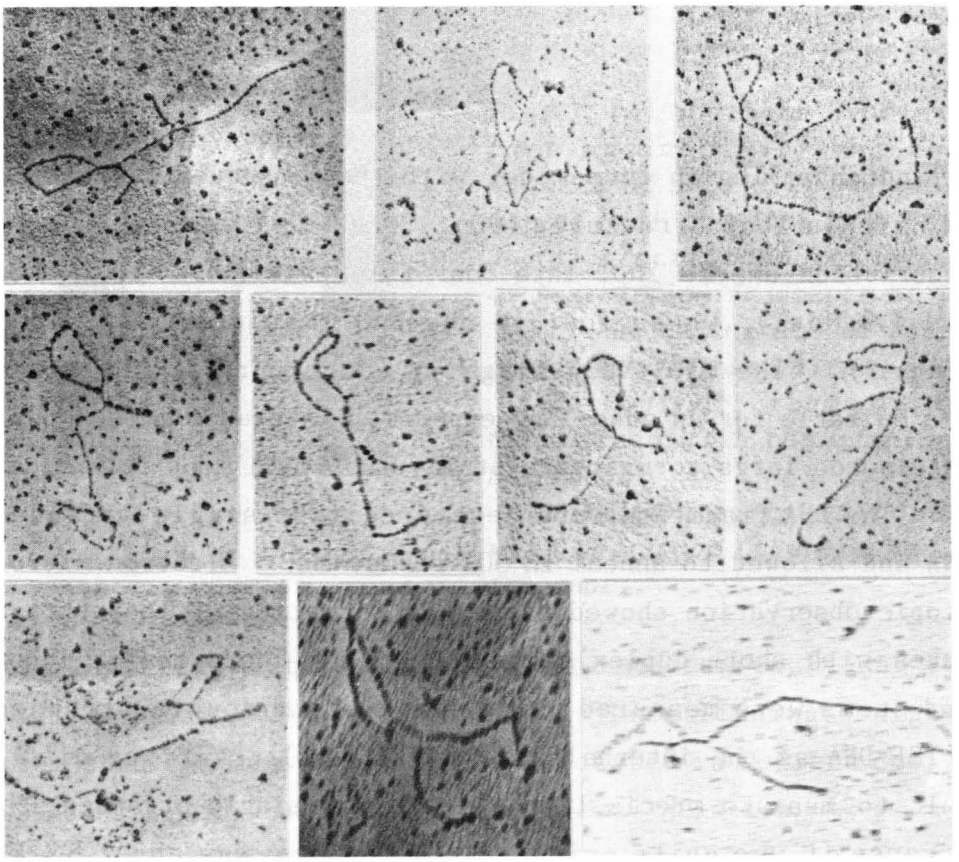


Fig. 4. Electron Micrographs of Loop Structures of DNA formed by Intrastrand Annealing.

Annealing was done in 50% formamide.

this result indicates that inverted repeat sequences of about 1 kb are present after an interval of about 5.4 kb. The interval may correspond to the span (3 Mdal) of the deletion.

These results suggest that inverted repeat sequences are implicated in the 3-Mdal deletion. That the H-2 gene was put between inverted repeat sequences suggests strongly the possibility that the H-2 gene is a transposon.

SUMMARY

The presence of inverted repeat sequences at both end-points of the 3-Mdal deletion of pUO1 was demonstrated by Southern hybridization and intra-strand annealing analysis. The sequences, which were about 1 kb in length, appeared to be implicated in the deletion. Such a DNA structure as the inverted repeat sequences with the H-2 gene between supported the possibility that the H-2 gene is a transposon.

CHAPTER IV

REARRANGEMENT OF PLASMID PUO1

Section 1
Molecular Modification of Plasmid pUO1 Caused by Deletion in a Foreign Host

Plasmid pUO1 is transmissible into several strains of Pseudomonas species and Escherichia coli and can express its phenotypes in these hosts. I mentioned before that pUO1 frequently loses the H-2 function coincidently with the loss of a specific segment of approximately 3 Mdal. The deletion plasmid was called pUO11. This deletion occurred more frequently in E. coli (about half the population harbored the deletion plasmid after overnight cultivation in L-broth) than in Moraxella sp. and Pseudomonas sp. (about 1 - 2% of the population). Other deletion mutants have not been detected in these hosts.

However, when pUO1 was conjugatively transferred into another strain of Pseudomonas sp., a new deletion mutant appeared. In this section, the characteristics of this deletion plasmid are described, and the composition of pUO1 DNA is speculated upon.

MATERIALS AND METHODS

Organisms and plasmids. Pseudomonas sp. E isolated from soil is a glycolate-assimilable bacterium that does not harbor plasmids. The taxonomical and resistance properties of this strain are listed in Tables I and II. Moraxella sp. B186, an auxotrophic (ilv^-) mutant of Moraxella sp. B harbor-

TABLE I. Taxonomical Properties of Pseudomonas sp. E

Rods (0.5 - 0.8 by 2 - 3 μm), motile by polar flagella.
Gram negative.
Colonies pale yellow, fluorescent pigment not produced.
Organic growth factors not required.
Obligately aerobic. Denitrification negative.
Nitrite formed from nitrate.
Glucose, gluconate, citrate, succinate, malate, glycolate, p-hydroxybenzoate and L-arginine utilized as carbon sources.
Glycerol and acetate not assimilated.
Ammonium salts, nitrate salts and amino acids utilized as nitrogen sources. Gelatin not liquefied.
Oxidase positive, catalase positive, urease positive.
Indol not produced. H_2S produced.

TABLE II. Resistant Properties of Pseudomonas sp. E

MIC is the minimum inhibitory concentration at which the organism can not grow in L-broth at 30°C for 24 hr.

Drug	MIC (μg/ml)
$HgCl_2$	10
Penicillin G	300
Ampicillin	300
Tetracyclin	10
Kanamycin	10
Rifampicin	5
Nalidixic acid	10

ing pUO1, was described in Chapter II.

Conjugation. The culture and mating conditions of donor and recipient cells were as described in Chapter II. The minimal fluoroacetate plate was used to select transconjugants.

Transformation. Transformation of Pseudomonas E was done using the method of Chakrabarty et al. (91).

Isolation and analyses of plasmid. Plasmid DNA was extracted by the method of Hansen and Olsen (68) and purified by CsCl-EtBr equilibrium centrifugation as described in Chapter II. Electron microscopy, agarose gel electrophoresis, and digestion with restriction endonucleases were done by the methods described in Chapter II. The molecular size of the plasmid was calculated from the contour length of ocDNA using ColE1 DNA (4.2 Mdal) as an internal standard.

RESULTS

Conjugal transfer of pUO1 to Pseudomonas sp. E

Cells of Pseudomonas sp. E (9×10^9/ml) grown in peptone medium were allowed to mate with cells of Moraxella sp. B186 (ilv^-, pUO1) (10^9/ml) for 2 hr in peptone medium, and transconjugants were selected on a minimal fluoroacetate plate. Fluoroacetate-assimilable pseudomonad clones were obtained at a frequency of 10^{-7} per donor cell. They all expressed the H-1 function, while one-third of them lacked the H-2 function and another third lacked both the H-2 function and mercury resistance. Analysis of their plasmid DNA by agarose gel electrophoresis revealed that the mutants deficient in H-2 harbored pUO11 and that the mercury- sensitive, H-2-deficient mutants harbored two small plasmids other than pUO1 or pUO11 (Fig. 1). The smaller plasmid, called pUO12, was present in larger amounts than the larger one, called pUO13.

Molecular sizes of deletion plasmids pUO12 and pUO13

Plasmid DNA of pUO12 and pUO13 was purified by CsCl-EtBr

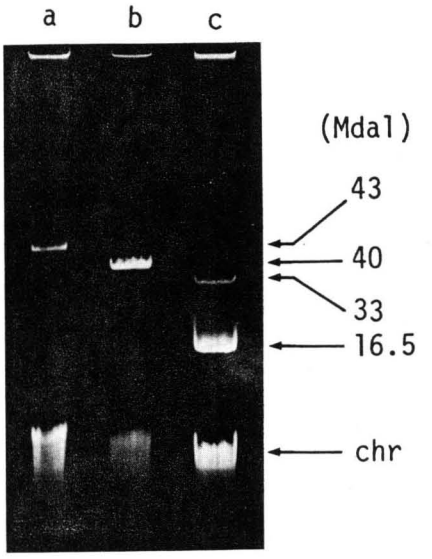


Fig. 1. Agarose Gel Electrophoresis of pUO1 and Its Deletion Derivatives.

Plasmid DNA was electrophoresed in a 0.8% agarose vertical slab gel under the conditions described in Chapter II. Sizes of plasmids were determined by measuring their relative mobilities against reference plasmids. Lane a, a clone carrying pUO1; lane b, a clone carrying pUO11; lane c, a clone carrying pUO12 and pUO13. chr, chromosomal DNA fragments.

equilibrium density gradient centrifugation and observed by electron microscopy (Fig. 2). From the contour length of the ocDNA, the sizes of pUO12 and pUO13 were determined to be 16.5 and 33 Mdal, respectively, on the average. These values agreed with the values estimated from the electrophoretic mobility (Fig. 1).

Transformation of Pseudomonas E with pUO12

When purified pUO12 was introduced into Pseudomonas E by the transformation technique, this organism became able to produce the H-1 enzyme. This suggests that pUO12 carries the H-1 gene.

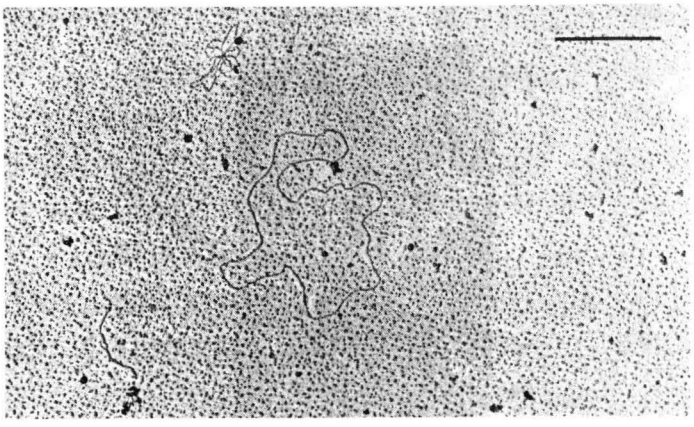


Fig. 2. Electron Micrograph of ocDNA of pUO12. The bar represents a length of 1 μm.

Formation of oligomers of pUO12 in Pseudomonas E

It was assumed that pUO13 is a dimer of pUO12 DNA because of the following observations: 1) pUO13 was twice the size of pUO12, 2) pUO12 and pUO13 gave identical patterns of products of restriction endonuclease cleavage, and 3) Pseudomonas E transformed with the purified pUO12 DNA still harbored pUO13 together with pUO12. When a larger amount of plasmid DNA was subjected to electrophoresis, plasmid bands corresponding to a trimer and a tetramer of pUO12 were observed as well as the bands of pUO12 and pUO13, although the oligomer bands were still less dense than the dimer band (data not shown).

Cleavage map of pUO12

pUO12 DNA was analyzed by digestion with the restriction enzymes BamHI, SmaI, SalI, EcoRI, and HindIII, whose cleavage sites on pUO1 and pUO11 were already mapped. SmaI did not cleave pUO12 DNA, but BamHI cleaved one site. Products generated by digestion with SalI, EcoRI, and HindIII are

TABLE III. Restriction Products from pUO11 and pUO12

Figures show fragment sizes in Mdal and letters denote names of pUO11 fragments designated in the previous section.

SalI			EcoRI			HindIII		
	pUO11	pUO12		pUO11	pUO12		pUO11	pUO12
A	14.8		A	15.0		A'	17.8	
B	9.6				11.1	B	17.8	
C'	8.2	8.2	B	9.6				16.0
		3.4	C	6.6		D	1.48	
D	2.8	2.8	D	3.1		E	0.60	0.60
E	2.2		F	2.4	2.4			
H	0.95	0.95	G'	1.35	1.35			
I	0.76	0.76	H	1.17	1.17			
			I	0.50	0.50			

listed in Table III. Each product of pUO12, with one exception in each digestion, was identical in size with one of the pUO11 products. This indicates that pUO12 is a deletion mutant derived from pUO11 DNA.

In general, a deletion mutant should generate one new cleavage fragment formed by conjunction of DNA left over after deletion. Therefore, a cleavage map of pUO12 could be brought from a pUO11 map by putting the new fragment in place of the pUO11 fragments missing in pUO12 (Fig. 3). This map was in conformity with the data of double digestions of pUO12 (Table IV).

DISCUSSION

Here, it was shown that the structure and functions of plasmid pUO1 was variable and that the DNA was modified in

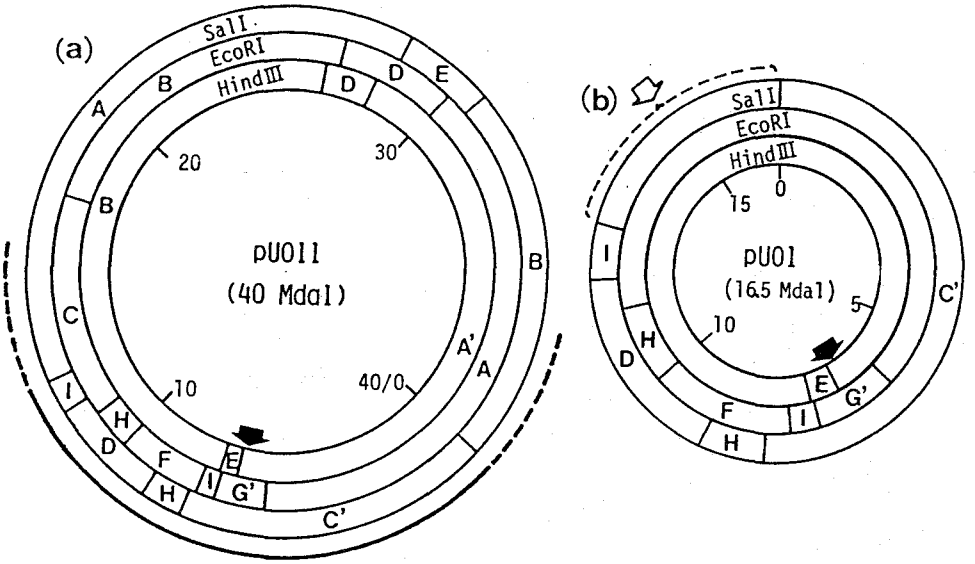


Fig. 3. Cleavage Maps of pUO11 (a) and pUO12 (b) for SalI, EcoRI, and HindIII.

Letters denote the fragments given in Table III. The arc outside the pUO11 map shows the region of pUO12 DNA (the dotted line indicates an indefinite boundary). Arrows ⬇ and ⇩ indicate the junction points after the deletions of the 3-Mdal and the 23.5-Mdal segments, respectively. The SalI site between fragments C' and B is used as a reference point. The size of the circle does not correspond to the actual size.

TABLE IV. Fragments Produced by Double Digestion of pUO12 with SalI, EcoRI and HindIII

Enzyme	Sizes (Mdal) of fragments									
	a	b	c	d	e	f	g	h	i	j
SalI-EcoRI	6.5	3.4	1.35	1.17	1.07	0.95	0.76	0.50	0.50	0.35
EcoRI-HindIII	11.1	2.4	1.17	0.80	0.58	0.45	ND			
HindIII-SalI	6.8	3.4	2.8	0.95	0.76	0.65	0.60			

ND, fragment not detected, but its presence can be predicted.

foreign hosts, becoming pUO11, pUO12, or its oligomers.

Whereas pUO1 and pUO11 were transmissible, pUO12 and its oligomers did not transfer themselves conjugatively from Pseudomonas E to other Pseudomonas strains (data not shown). Therefore, pUO12 presumably lacks a transfer gene carried on pUO1 or pUO11. I suppose that pUO12 was formed from pUO1 via two steps of deletion (Fig. 4); the first is the deletion of a segment of about 3 Mdal containing the H-2 gene, resulting in pUO11, and the second is the deletion of a segment of about 23.5 Mdal containing genes for mercury resistance and transfer function, generating pUO12. pUO12 readily becomes a dimer. The first deletion frequently occurs in Moraxella and some foreign hosts, especially in E. coli, but the second deletion has never been detected in any hosts other than Pseudomonas sp. E.

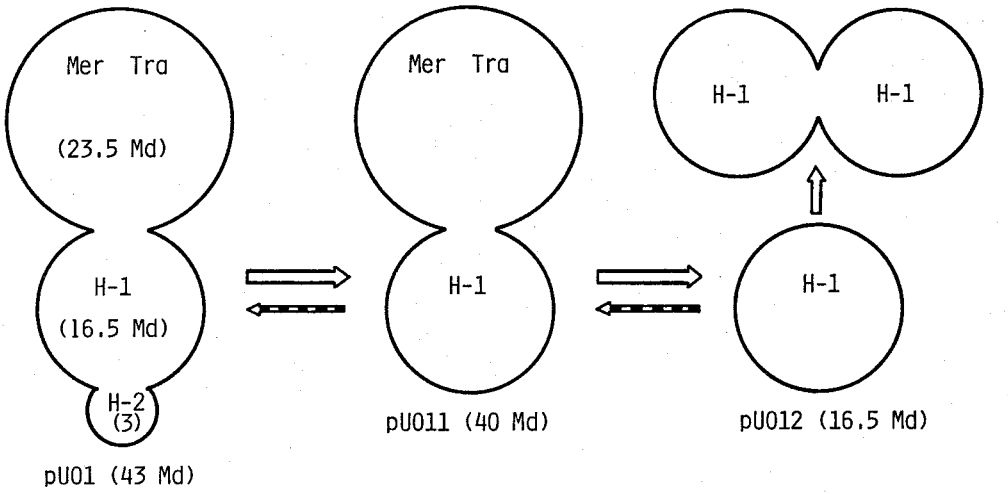


Fig. 4. Formation of pUO12 via Two Steps of Deletion.
Mer and Tra represent mercury resistance and transfer functions, respectively.

Viewed from the reverse of the successive deletion of pUO1, the formation of plasmid pUO1 may be considered; pUO12 might be a original replicon of pUO1, which carries an autonomously replicating function and the H-1 gene. pUO12 fused with a 23.5-Mdal DNA segment encoding for mercury resistance and transfer function, and then with a 3-Mdal segment containing the H-2 gene. It has been mentioned that the 3-Mdal segment may be a transposon.

Pseudomonas E seems unacceptable to Moraxella plasmid pUO1, so that pUO1 introduced into a Pseudomonas cell might be either eliminated or modified to remain stable. When pUO1 was introduced into Pseudomonas E by transformation, transformants containing pUO1 or pUO11 were not obtained; all transformants carried pUO12, which was maintained stably if the cells were grown in medium supplemented with fluoroacetate. However, pUO1 transferred by conjugation was sometimes maintained stably in Pseudomonas E, and from the transconjugant, pUO12-carrying progeny did not appear. I don't know why pUO1 is stable in these clones. It is possible that pUO1 DNA was stabilized by a slight modification. However, even if pUO1 DNA was isolated from the stable clone and introduced into the wild Pseudomonas E, pUO1 was not stably maintained. Therefore, the host cell being a mutant deficient in restriction functions might account for the stable maintenance of pUO1 in Pseudomonas E.

SUMMARY

When pUO1 was introduced into a glycolate-assimilable isolate, Pseudomonas sp. E, a new deletion plasmid, called pUO12, occurred relatively frequently. Its size was estimated to be 16.5 Mdal by measuring the contour length of

ocDNA on an electron micrograph. Restriction analysis located pUO12 DNA on the pUO1 map, and so the map of pUO12 could be constructed. pUO12 encoded the H-1 gene but lacked the genes for H-2, mercury resistance, and transfer function.

Section 2

In Vivo Recombination Between pUO1 and Multiple-Resistant Plasmid RP4 in *Pseudomonas*

Rearrangement of genes by DNA recombination has played an important role in the evolution of microorganisms. In particular, illegitimate recombinations, including transposition, deletion, duplication, and cointegration, promote the diversification of plasmids as well as of microorganisms. The rearrangement of plasmids by recombination may be common in nature, but the recombinant plasmids will not survive unless there is suitable selection pressure. If such conditions are created in a laboratory, *in vivo* recombination may cause more rapid evolution of plasmids than in nature, and is therefore useful in the creation of new plasmids. Chakrabarty *et al.* (92) used the technique of "plasmid-assisted molecular breeding" to build up bacterial strains capable of degrading chlorinated herbicide 2.4.5-T.

Plasmid pUO1 often loses a certain segment (about 3 Mdal) containing the H-2 gene, which is bounded by inverted repeats of about 1 kb, suggesting that the segment may be transposable. Certain Hg^r determinants are known to reside on transposons such as Tn501 (93) and Tn2613 (94). So to see if the H-2 and Hg^r determinants on pUO1 are transposable, plasmid RP4 used as a recipient replicon was allowed to coexist with pUO1 in a *Pseudomonas* species. There was frequent recombination between pUO1 and RP4.

In this section, I describe this event as an example of the rearrangement of plasmid genes.

MATERIALS AND METHODS

Organisms, plasmids, and phages. Pseudomonas sp. E harboring pUO1 was described in the previous section. E. coli 20SO (thi lac) harboring RP4 was a kind gift of Dr. T. Nakazawa (95). Plasmid RP4 has a broad host range and a size of 39 Mdal, and it encodes the determinants for resistance to kanamycin (Km^r), tetracycline (Tc^r), and ampicillin (Ap^r). E. coli C600 (rec^- nal^r) was used as a recipient cell for mating out new recombinant plasmids from transconjugants. Phages PRD1 and PRR1, which specifically adsorb to the pili produced by plasmids of IncP1 group (96), were used to determine the tra gene of recombinants. Pseudomonas aeruginosa PU21 (RP4, ilv str^r rif^r) was used as a host cell for phages PRD1 and PRR1.

Conjugation. Mating was done using the centrifugation method of Stuy (84). The minimal chloroacetate medium used for the selection plate was described in Chapter I.

Assay of the H-1 and H-2 enzymes and drug resistance. The dehalogenase activity of cells was measured using toluenized cells as described in Chapter III. Drug resistance was determined by growth on peptone plates containing 30 μg of $HgCl_2$, or 50 μg of kanamycin, tetracycline, or ampicillin, per milliliter.

Isolation and analyses of plasmid. Procedures for isolation of plasmids, agarose gel electrophoresis of DNA, electron microscopy of ocDNA, and digestion of plasmid with restriction endonucleases were described in Chapter II.

Plaque formation. Phage was increased by cultivating with P. aeruginosa PU21, and an adequate amount of phage was mixed with freshly grown cells harboring RP4, pUO1, or a recombinant plasmid. The mixture was suspended in soft agar (L-broth) kept at 50°C, and poured on a hard agar plate (L-broth). The plate was incubated at 30°C for 24 hr.

RESULTS AND DISCUSSION

Recombination between pUO1 and RP4

To allow RP4 to coexist with pUO1, Pseudomonas sp. E harboring pUO1 was mated with E. coli 20S0 harboring RP4, and transconjugants were selected by plating on either minimal chloroacetate agar containing kanamycin (20 μg/ml), or nutrient agar containing both $HgCl_2$ (30 μg/ml) and kanamycin (50 μg/ml).

Transconjugant colonies, which were all Pseudomonas, appeared at the frequency of 10^{-5} per donor E. coli cell in both media. The colonies were expected to carry both pUO1 and RP4. However, when the colonies were transferred onto fresh selection plates to be purified, many of them no longer grew. Some 100 of the transconjugants purified were tested for phenotype. About half had all of the markers of pUO1 (H-1 H-2 Hg^r) and RP4 (Km^r Tc^r Ap^r), but the pUO1 markers were rapidly lost in subsequent cultivation. Another 30% had RP4 markers alone and the remaining 20% lacked one or two markers. On the basis of phenotype, the transconjugants were classified into the six groups shown in Table I.

Electrophoresis of plasmids showed that Group 1 carried pUO1 and RP4, while Group 2 carried RP4 alone. Groups 3 to 6 carried single plasmids different in size from either pUO1 or RP4, but similar among members of one group; that of Group

TABLE I. Phenotypes of Transconjugants Obtained by Mating Pseudomonas E (pUO1) with E. coli 20S0 (RP4)

Strain		Phenotype						Plasmid
		H-1	H-2	Hg^r	Km^r	Tc^r	Ap^r	
Pseudomonas E (pUO1)		+	+	+	−	−	+*	pUO1
E. coli 20S0 (RP4)		−	−	−	+	+	+	RP4
Transconjugants:								
Group	Selection**							
1	A and B	+	+	+	+	+	+	pUO1+RP4
2	A and B	−	−	−	+	+	+	RP4
3	A	+	+	−	+	+	+	pDR1***
4	B	−	−	+	+	+	+	pDR2***
5	A	+	+	+	+	−	+*	pDR3***
6	A	−	+	−	+	+	+	pDR4***

* The resistance was due to the Pseudomonas host. It was verified by mating out to an Ap^S host.

** A; a chloroacetate plate containing kanamycin (20 μg/ml).
B; a peptone plate containing $HgCl_2$ (30 μg/ml) and kanamycin (50 μg/ml).

*** A representative plasmid of each group.

3 was about 48 Mdal, Group 4, 45-47 Mdal, and Groups 5 and 6, about 44 Mdal. By transferring the new plasmids into E. coli C600, it was confirmed that the plasmids mediated the phenotypes of individual transconjugants, except that the Ap^r of Group 5 was due to the Pseudomonas host.

The new plasmids and their phenotypes were stably inherited in Pseudomonas E and E. coli even under nonselective conditions. However, pUO1 coexisting with RP4 was very unstable. When a transconjugant harboring both pUO1 and RP4 was serially grown in unsupplemented nutrient broth twice, such new plasmids appeared in 5% of the progeny, although 90% carried RP4 alone. These facts suggest that the new plasmids

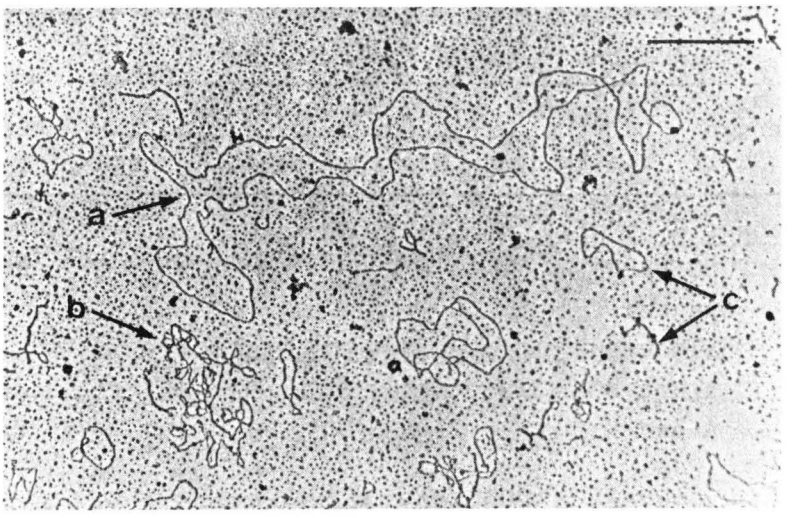


Fig. 1. Electron Micrograph of an ocDNA Molecule of pDR1. The bar represents a length of 1 um. a, ocDNA; b, ccDNA, c, Col E1 DNA.

are recombinants between pUO1 and RP4, but that the DNA molecules are not intact cointegrates of the two plasmid DNA.

DNA composition of recombinant plasmids

Representative plasmids for individual groups (pDR1 in Group 3, pDR2 in Group 4, pDR3 in Group 5, and pDR4 in Group 6) were purified and then observed by electron microscopy (Fig. 1). From the contour length of ocDNA, the size of recombinant plasmid was calculated: pDR1 was 48 Mdal; pDR2, 47 Mdal; pDR3, 44 Mdal; and pDR4, 44 Mdal. Purified DNA was analyzed using five restriction enzymes. With one or two exceptions, the restriction fragments from these plasmids were identical with fragments of pUO1 or RP4 (Table II). By comparison with the restriction maps of pUO1 and RP4 (97), these recombinant plasmids were mapped (Fig. 2), and the portion of pUO1 and RP4 DNA in the hybrid molecule were

TABLE II. Restriction Fragments of Recombinant Plasmids

	pDR1					pDR2				
	HindIII	BamHI	SmaI	SalI	EcoRI	HindIII	BamHI	SmaI	SalI	EcoRI
A	29	36	18	13.8**	38	39	24	14.3**	30	24
B	14.6	5.8	12.3	12.4	3.0*	6.2	24	10.7**	13.8**	21
C	3.0*	2.2*	10.7**	8.2*	2.4*	1.48*		9.6**	2.5	3.1*
D	0.60*	1.22*	4.3**	6.1	1.35*			5.1		
E			0.89*	2.8*	1.17*			3.9		
F			0.50*	2.2*	0.50*			2.8*		
G				1.20*				0.40**		
H				0.95*						

	pDR3					pDR4				
	HindIII	BamHI	SmaI	SalI	EcoRI	HindIII	BamHI	SmaI	SalI	EcoRI
A	17.8*	23	18.6	14.8*	18.6	25	33	18.0	13.8**	39
B	16.6	17.8*	12.6*	12.6	9.6*	17.8	2.2*	10.7**	12.7	3.1*
C	3.0*	2.2*	7.4*	8.2*	6.6*	3.0*	1.66	8.0	8.2*	1.35*
D	2.8	1.22*	4.1	2.8*	3.0*	0.60*	1.22*	4.3**	5.9	0.50*
E	1.48*		0.89*	2.2*	2.4*			0.89*	2.2*	
F	0.60*		0.50*	1.20*	1.35*			0.50*	1.20*	
G				0.95*	1.17*					
H				0.76*	0.50*					

Fragments are represented in size (Mdal).
* fragment identical with that of pUO1.
** fragment identical with that of RP4.

roughly located by also taking into consideration of the gene loci (Fig. 3). Junctions were not precisely defined.

pDR2 formed by incorporation of Hg^r determinant

An Hg^r recombinant, pDR2, was composed of what seemed to be the complete RP4 DNA and a 7.6-Mdal pUO1 segment carrying an Hg^r determinant. To see if the inserted segment was a transposable unit, several recombinant plasmids independently isolated with the phenotype H-1^- H-2^- Hg^r Km^r Tc^r Ap^r were analyzed in the same way. In every plasmid, small pUO1 segments carrying the Hg^r determinant were incorporated in

the same site of RP4 molecules, as in the case of pDR2. However, the segments were of three sizes: 7.6, 5.5, and 5.3 Mdal. The segments seemed identical at one end and different in length. The 5.5-Mdal segment was found to be inserted in an orientation opposite to the others. It was reported that the Tol^+ phenotype of the TOL plasmid is transposable to

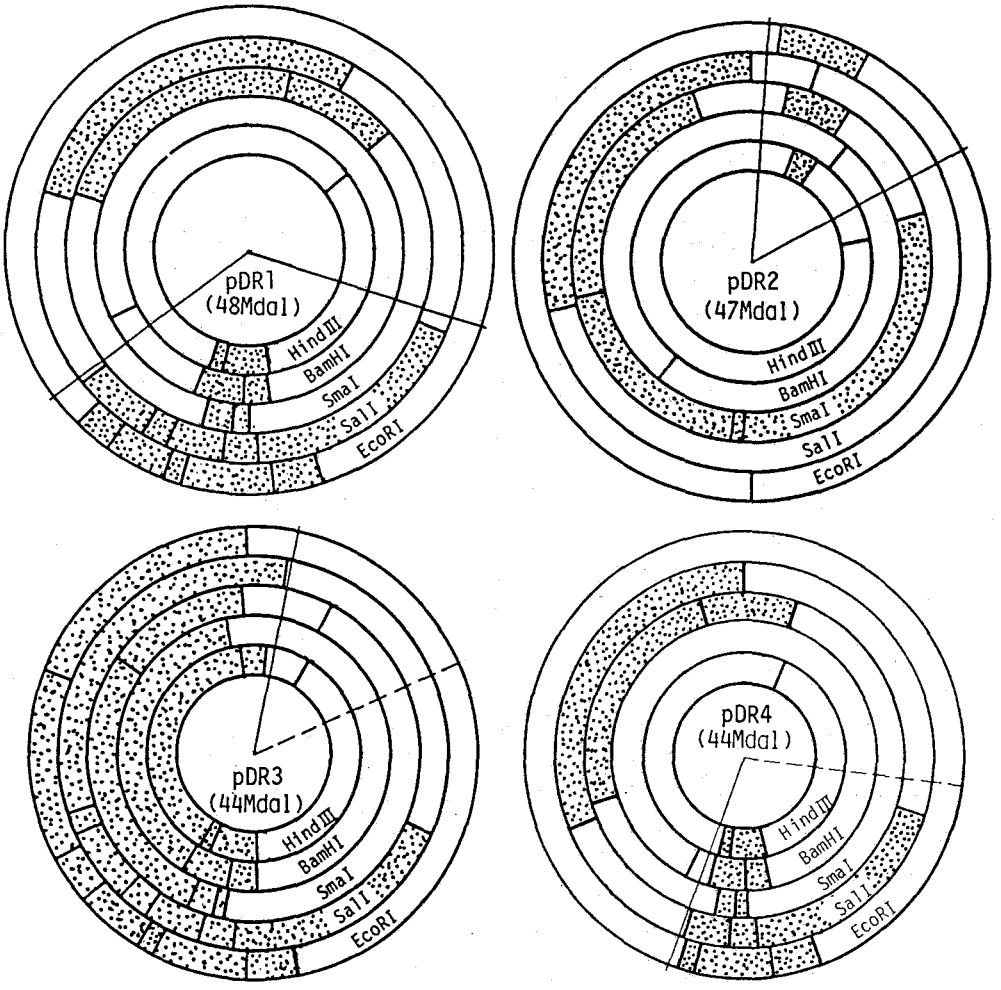


Fig. 2. Cleavage Maps of the Recombinant Plasmids.
The stippled fragments are identical with the fragments generated from pUO1 or RP4.

various R plasmids, but that its transposition does not involve a unique segment (98). Therefore, it seems reasonable that the formation of pDR2 or analogous recombinants should involve transposition of the Hg^r determinant from pUO1.

It is not clear if the 3-Mdal segment carrying the H-2 gene is a transposable element, because incorporation of the segment into RP4 did not occur here. The segment was readily deleted from pDR1, pDR3, and pDR4, as from pUO1.

Mechanism proposed for the formation of pDR1, pDR3 and pDR4

These recombinant plasmids other than pDR2 seem formed by a different mechanism. pDR1 (48 Mdal) and pDR4 (44 Mdal) contain a similar, and possibly identical, RP4 segment (about 32 Mdal) and different amounts of pUO1 DNA. One is a segment of about 16 Mdal carrying the H-1 and H-2 genes and the other is about 12 Mdal carrying the H-2 gene. pDR3 (44 Mdal)

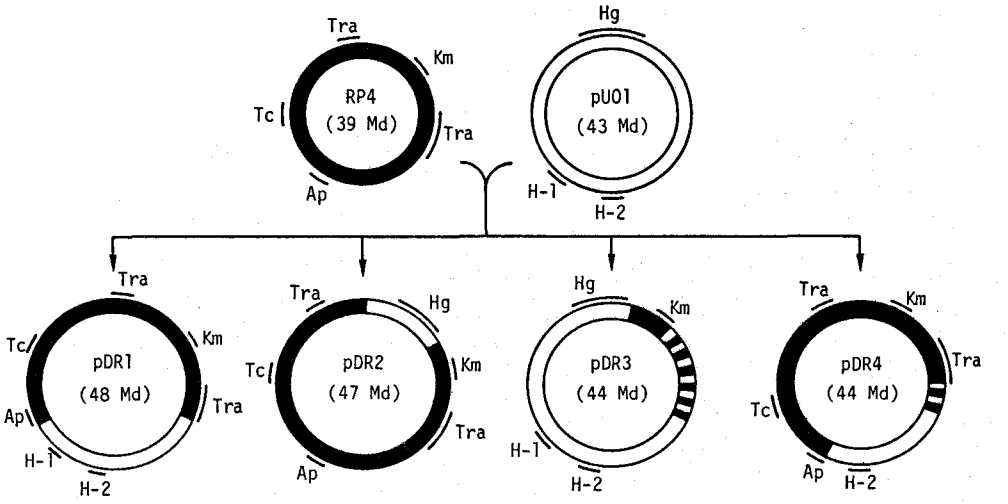


Fig. 3. DNA Composition of the Recombinant Plasmids. The black area indicates RP4 DNA and the white area, pUO1 DNA. The striped areas are still unidentified.

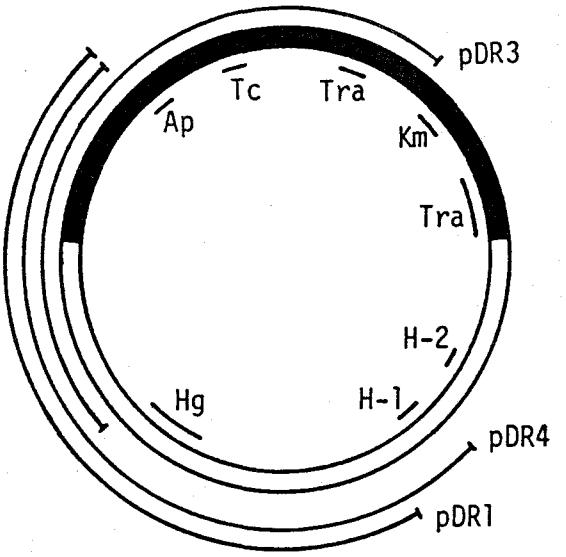


Fig. 4. Tentative Mechanism for Formation of the Recombinants pDR1, pDR3, and pDR4.

RP4 DNA (black) and pUO1 DNA (white) cointegrate intactly at a specific site of each DNA to form one transient large molecule, from which the different segments indicated by the arcs outside the circle are deleted.

consists of the large pUO1 DNA and the small RP4 DNA carrying the Km^r gene. Upon electrophoresis of plasmid samples prepared from Group 1 transconjugants, a faint plasmid band corresponding to about 80 Mdal, in addition to the bands of pUO1 and RP4, often appeared. This large DNA molecule and pUO1 readily disappeared in serial cultivation and occasionally the recombinant plasmids mentioned above appeared. These observations suggested that the mechanism for the formation of pDR1, pDR3, and pDR4 involved, first, intact DNA of pUO1 and RP4 joining in a transient large molecule, from which the different segments shown in Fig. 4 were deleted.

Transfer function of recombinant plasmids

All recombinant plasmids were transmissible to Pseudomonas spp., E. coli, and Moraxella sp. and could express dehalogenase or mercury resistance analogously to pUO1 in these hosts. To see if the transfer functions of these recombinant plasmids were from pUO1 or from RP4, phages PRD1 and PRR1 were used as probes that specifically adsorb to the pili produced by RP4. Recombinants pDR1, pDR2, and pDR4 conferred susceptibility to these phages upon the host cells, while pDR3 and pUO1 did not. This implies that the functional tra genes in pDR1, pDR2, and pDR4 were derived from RP4, and that the gene in pDR3 was derived from pUO1. Thus the H-1, H-2, or Hg^r gene of pUO1 with a limited host range acquired the transfer function of plasmid RP4 with a broad host-range. This may contribute to the dissemination of the dehalogenase and mercury reductase among many other Gram-negative bacteria.

Here, four recombinant plasmids were obtained, but additional kinds might be obtained if the selection pressure were changed. The formation of these recombinants seems to be due to site-specific recombination, although it is not clear whether it is independent of the host recombination system. I think that the frequent recombination observed here is not necessarily due to characteristics peculiar to pUO1 or RP4. Plasmids are variable and their genetic rearrangement is not rare in nature.

SUMMARY

When plasmid pUO1 (43 Mdal) was allowed to coexist with R-plasmid RP4 (39 Mdal) in a strain of Pseudomonas, genetic

exchange between both plasmids frequently occurred. Four recombinant plasmids with different phenotypes were obtained, and their DNA compositions were analyzed. They were all in the range of 44 - 48 Mdal and were not intact cointegrate molecules. Formation of these hybrids was probably caused by site-specific recombination between pUO1 and RP4, followed by various deletions. These recombinant plasmids were inherited stably in E. coli and Pseudomonas sp. By this rearrangement, the dehalogenase genes acquired a broad host-range transfer function.

CONCLUSION

Halogenated organic compounds generally resist microbial attack. Fluoroacetate, an extremely toxic chemical, has a stable carbon-fluorin bond. I isolated three bacterial strains, Pseudomonas sp. A, Moraxella sp. B, and Pseudomonas sp. C, from industrial waste-water of Osaka and Saitama, that could utilize fluoroacetate as the sole carbon source. These organisms had haloacetate dehalogenase, which cleaved carbon-halogen bonds as follows: $XCH_2COOH + H_2O \longrightarrow HOCH_2COOH + HX$ (X, halogen). I found that the production of the dehalogenases of Moraxella sp. B was mediated by a plasmid, named pUO1. In this investigation, two kinds of haloacetate dehalogenases were purified and characterized, and the structure and the functions of plasmid pUO1 were analyzed.

I. Haloacetate dehalogenases H-1 and H-2.

Pseudomonas sp. A produced dehalogenase H-1 constitutively, and the enzyme preferentially acted on fluoroacetate, while Moraxella sp. B produced two dehalogenases; an inducible one, which was the same enzyme as Pseudomonas H-1, and a constitutive one named H-2, which acted on chloroacetate, bromoacetate, and iodoacetate, but not on fluoroacetate. The H-1 and H-2 enzymes were purified from Pseudomonas sp. A and Moraxella sp. B, respectively, to a homogeneous state; the H-1 enzyme was cristallized.

Physicochemical and catalytic properties of the H-1 and H-2 dehalogenases were studied. The molecular weights were 42,000 and 43,000, $s^{o}_{20,w}$ was 5.2 S and 4.1 S, pI was pH 5.4 and 5.2, respectively. The amino acid compositions of the two enzymes did not resemble each other. H-1 was specifically active against haloacetates. Of the haloacetates

tested, monofluoroacetate was most rapidly dehalogenated (Km; 2 mM), but monoiodoacetate was scarecely. H-2 was active against monochloroacetate (Km; 2.5 mM), monobromoacetate (Km; 0.5 mM), and monoiodoacetate (Km; 1.1 mM) but inert against monofluoroacetate. H-2 had a little activity against 2,2-dichloroacetate and 2-chloropropionate but no activity toward trichloroacetate or 3-chloropropionate. Both enzymes were very sensitive to inhibition with thiol reagents such as pCMB. The optimum pH and temperature were pH 9 - 9.5 and 50°C for both enzymes.

II. Plasmid pUO1 that determines haloacetate dehalogenases.

Moraxella sp. B harbored a plasmid, pUO1, which was cured with mitomycin C coincidently with the disappearance of the H-1 and H-2 activities and mercury resistance. The plasmid when introduced into the cells of the cured Moraxella or Pseudomonas acidovorans conferred the activities of H-1 and H-2 and mercury resistance on these hosts. These observations meant that pUO1 determined the H-1 and H-2 enzymes and mercury resistance. pUO1 lost spontaneously a segment of about 3 Mdal coincidently with the loss of the H-2 function, generating a deletion plasmid, pUO11. pUO1 and pUO11 were transmissible to Pseudomonas spp. and E. coli, and expressed their functions in these hosts.

The molecular sizes of pUO1 and pUO11 were estimated to be 43.7 and 40.1 Mdal, respectively, by measuring contour lengths of ocDNA. Restriction maps of pUO1 and pUO11 were constructed for BamHI, SmaI, HindIII, EcoRI, and SalI, and by comparing the two maps, the locus of the 3-Mdal deletion was determined.

The H-1 and H-2 genes were cloned onto plasmid pBR322 in E. coli, and by identification of the cloned DNA fragments,

the loci of the H-1 and H-2 genes on the pUO1 map were determined. When the promoter of the H-1 gene cloned was exchanged for the promoter of the Tc^r determinant of pBR322, quantitative expression of the H-1 gene increased about three-fold in E. coli.

Two kinds of hybrid pBR322 containing the H-1 gene were maintained stably in E. coli, while three kinds of hybrids containing the H-2 gene were all so unstable that they readily disappeared or changed to various deletion mutants during serial cultivation. Enzymatic excision of a specific segment from an inserted pUO1 fragment resulted in stabilizing the unstable H-2 hybrid. The excised segment corresponded to the area where one end-point of the 3-Mdal deletion was located. Southern hybridization analysis showed that repeated sequences were present at both end-points of the 3-Mdal deletion. Furthermore, it was demonstrated by intra-strand annealing analysis that the sequences were inverted repeats of about 1 kb. The H-2 gene put between inverted repeat sequences seemed to be a transposon. However, the transposition of the H-2 gene has never been seen.

Although the H-1 and H-2 enzymes were similar in molecular weight and other properties, there was no homology between the base sequences of the two genes. This suggested that there was no ancestral relationship between the H-1 and H-2 enzymes.

Plasmid pUO1 was very variable in foreign hosts, and rearrangement of the plasmid genes occurred frequently. When pUO1 was transferred into E. coli, pUO1 often became the deletion plasmid pUO11 by losing a segment of about 3 Mdal containing the H-2 gene. When pUO1 was introduced into a Pseudomonas strain, another deletion plasmid, pUO12, occurred in addition to pUO11. pUO12 (16.5 Mdal) did not have

transfer function and carried the H-1 gene alone.

When pUO1 was allowed to coexist with R-plasmid RP4 (39 Mdal) in a Pseudomonas strain, recombination between the two plasmids occurred frequently. Four recombinant plasmids with different phenotypes and DNA compositions were obtained and they were inherited stably in both E. coli and Pseudomonas sp. By this rearrangement, the dehalogenase genes acquired the broad-host-range transfer function of RP4.

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